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Synthesis and Inactivation of Bacterial Luciferase Determined by Immunochemical Techniques

COMPARISON WITH TOTAL PROTEIN SYNTHESIS AND TURNOVER*

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The luciferase of the luminous marine bacterium *Vibrio harveyi* is rapidly inactivated during late log phase and stationary phase. An analysis of the rates of synthesis and degradation of total protein and of luciferase throughout growth of the culture shows that the rate of total protein synthesis per cell was maximal in mid-log phase and declined steadily to a very low level in stationary phase. The synthesis of luciferase was maximal in early log phase and declined throughout log phase growth to near zero at about the time that luciferase inactivation began. Luciferase was stable throughout exponential growth and accumulated in the cells, but during stationary phase, luciferase cross-reacting material was lost from the 100,000 × *g* supernatant of cell extracts at the same rate as luciferase activity. Neutralization of luciferase activity in uncentrifuged lysates with antiluciferase antibody, however, suggested an accumulation of inactive cross-reacting material during stationary phase. The antibody was shown to complex and precipitate proteolytic fragments of luciferase produced *in vitro*. Furthermore, the antibody was shown to recognize chymotrypsin-treated luciferase as well as native luciferase in the luciferase activity neutralization assay.

Chloramphenicol blocked protein synthesis quite effectively while by comparison, cyanide acted very slowly and ineffectively to block protein synthesis. Cyanide blocked total protein turnover immediately while the effect of chloramphenicol upon protein turnover was not apparent until 1-2 h after addition. Even though cyanide blocked total protein turnover immediately, luciferase inactivation continued for about 1 h, and, as in the experiment without cyanide, the luciferase protein was lost from centrifuged lysates at the same rate as luciferase activity. Inactivation of bacterial luciferase appears to be associated with a decrease in solubility, such that the inactive protein is separated from the active luciferase in lysates by centrifugation.

The control of the biosynthesis of bacterial luciferase has been the subject of research for nearly 30 years (see Refs. 1 and 2 for reviews). The current model for control of the expression of luciferase and accessory enzymes required for luminescence (*e.g.* enzymes involved in synthesis of the aldehyde substrate) (3) is that the accumulation of a species-specific constitutively produced small molecule, the autoinducer, to a threshold level in the culture medium triggers the derepression of the luminescence system (4). The autoinducer of *Vibrio fischeri* has been isolated and its structure determined to be *N*-(3-oxohexanoyl)-3-aminodihydro-2(3*H*)-furanone (*i.e.* *N*-(β-ketocaproyl)homoserine lactone (5). The compound has been synthesized and shown to have biological activity. This system of autoinduction allows free living cells in the ocean to forego the synthesis of luciferase and light emission. Under high nutrient conditions, cell densities may be reached that allow expression of the luminescence (6). This type of control would be of definite selective advantage to the bacterium, since light emission occurs only at high cell densities, thereby yielding high light intensities (1, 2).

When *Vibrio harveyi*, as well as most other luminous bacteria, is grown on solid medium, luminescence is readily visible from the colonies as soon as the colonies are large enough to be seen by the unaided eye. After several days, however, the luminescence from the cells declines, and ultimately the cells become dark. Likewise, in liquid medium, the cells attain maximum luminescence per milliliter of culture in late log phase of growth (7), and the luminescence then decreases dramatically as the cells enter stationary phase of growth (8).

We have analyzed the process of loss of luminescence during late log phase and early stationary phase in some detail and have made the following observations (8). 1) The loss of luminescence is due to a loss of luciferase activity. 2) The apparent inactivation of luciferase requires the integrity of the cell; that is, even though the luciferase is exquisitely sensitive to inactivation by proteases (9-11), the activity is stable in crude lysates. 3) The inactivation process stops when the cells are deprived of oxygen. 4) If either chloramphenicol or cyanide is added to cells before the onset of inactivation, then the inactivation process never begins, but if either inhibitor is added after the onset of inactivation, the inactivation process continues unabated for 2-3 h before halting. 5) A nonconditional aldehyde-deficient mutant of *V. harveyi* does not inactivate luciferase during stationary phase, and a temperature-sensitive aldehyde-deficient mutant of *V. harveyi* does not inactivate luciferase during stationary phase when grown at the nonpermissive temperature but does inactivate luciferase during stationary phase when grown at the permissive temperature. 6) The ATP level in the wild type of *V. harveyi* is constant throughout log phase and perhaps decreases slightly in early stationary phase while the ATP level

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in the nonconditional aldehyde-deficient mutant increases about 2-fold as the cells enter stationary phase. Cyanide causes a rapid decrease in ATP while chloramphenicol causes a rapid increase in ATP; each causes a slowing or cessation of the inactivation 2–3 h later.

We undertook the experiments described in this paper to further evaluate this inactivation process. Our earlier experiments (8) did not address the question of luciferase degradation, but rather luciferase inactivation. Using immunochemical techniques, we have now measured the specific rates of luciferase synthesis relative to total protein synthesis throughout growth of the culture. We found that luciferase synthesis had essentially stopped before the inactivation process began. By monitoring the anti-luciferase cross-reacting material, we found that the luciferase protein disappeared from the 100,000 × *g* supernatant of cell lysates at the same rate as luciferase activity. However, a luciferase activity neutralization assay of uncentrifuged lysates suggested an accumulation of inactive antigen in the cells during stationary phase.

EXPERIMENTAL PROCEDURES[1]

Chemicals. Phosphate buffers were prepared by mixing appropriate volumes of 2 M stock solutions of NaH_2PO_4 and K_2HPO_4. Trizma base, Bis-Tris[2], disodium EDTA, bovine serum albumin (Fraction V), FMN, n-decyl[1] aldehyde (Grade I), and chloramphenicol were products of Sigma. Potassium cyanide and sodium fluoride were both Baker Reagent Grade. Dithioerythritol and glycine were products of Aldrich. All salts (unless otherwise noted) used in the preparation of bacterial growth media were Mallinckrodt A.R. Grade, glycerol was Fisher A.C.S. Grade, and Bacto-Agar and Freund's adjuvants (complete and incomplete) were products of Difco. Bacto-Tryptone (Difco) or casein hydrolysate (Gibco) and yeast extract (Difco or Gibco) were used to prepare complex media; no difference was noted in growth on media supplied by the two vendors. L-[4,5-^{3}H] leucine (specific activity 55 Ci/mmole) and nonradioactive L-leucine were purchased from ICN Pharmaceuticals, Inc.

Bacterial Growth Conditions. *Vibrio harveyi* strain 392 [formerly *Beneckea harveyi* (12); recently reclassified (13)] was used throughout. The medium used in most of our experiments was Bis-Tris minimal medium. This medium contained (per liter): NaCl, 11.7 g; KCl, 0.75 g; $MgSO_4 \cdot 6H_2O$, 12.3 g; $CaCl_2 \cdot 2H_2O$, 12.15 mg; K_2HPO_4, 1.0 g; NH_4Cl, 1.02 g; glycerol, 3.0 ml; ferric ammonium citrate, 1.4 mg; arginine, 0.5 g; and Bis-Tris, 10.46 g. To avoid precipitation of a number of salts, the medium was prepared by making stock solutions of the components, sterilizing and storing separately, and mixing just before use. The Bis-Tris and K_2HPO_4 were prepared together and adjusted to pH 7.3 before autoclaving. Bis-Tris complete medium was prepared by supplementing the minimal medium with Bacto-Tryptone (5 g/l) and yeast extract (3 g/l). Solid medium contained 12 g Bacto-Agar per liter Bis-Tris complete medium. Cultures were stored for as long as 6 months on tightly sealed slants of the medium.

Inocula for growth experiments were prepared by transferring cells from the slants to shaker tubes of the complete medium and allowing the cells to grow to early stationary phase with vigorous aeration. Such a culture could be used as an inoculum for an experiment to be done in complete medium. For experiments done in minimal medium, 10–20 µl of the stationary phase culture was transferred to a tube containing 10 ml of the minimal medium and the tube was shaken vigorously until the optical density of the culture at 660 nm (OD^{660}) reached 0.3 to 2.0. In all experiments, cultures were inoculated to an initial OD^{660} of 0.01.

Experimental cultures were grown in baffled Erlenmeyer flasks with shaking at 200 rpm in a New Brunswick Scientific Aquatherm Water Bath Shaker. To allow for vigorous aeration, the culture volume was never greater than one third the volume of the flask. This was very important because the growth rate and level of luminescence varied somewhat with the degree of aeration, especially in the minimal medium.

Cell densities during growth were determined by measuring the optical density of the culture at 660 nm on a Turner Model 330 Spectrophotometer. In Bis-Tris minimal medium, 1 OD^{660} unit = 1.7×10^9 cells/ml, and in the complete medium, 1 OD^{660} unit = 6.5×10^8 cells/ml. In both media, the pH of the culture remained between 6.9 and 7.3 throughout the experiment, and cell death did not occur in late stationary phase, as long as cultures were kept well-aerated.

Incorporation of ^{3}H-Leucine. Pulse-chase experiments were done by first incubating a culture at the desired cell density for 20 minutes with ^{3}H-leucine (1–2 µCi/ml). The culture was then centrifuged at 20,000 x g, 27°C, for 10 minutes. The cells were washed with fresh medium containing unlabeled L-leucine at 750 µg/ml and finally resuspended in medium from a parallel culture from which cells had been removed by centrifugation and to which unlabeled leucine (750 µg/ml) had been added. These manipulations produced very little lag in cell growth and luciferase production.

In the Bis-Tris minimal medium, at least 99% of the labeled leucine added was incorporated into trichloroacetic acid precipitable material in less than 0.5 minutes at 27-28°C at all cell densities at which labeling was done (OD^{660} = 0.1 to 2.5). The use of the minimal medium for these experiments was required because the incorporation of label was very poor from the complex medium, probably due to the abundance of leucine in the complex medium.

In order to measure rates of total protein synthesis and luciferase synthesis, it was necessary to supplement the ^{3}H-leucine with unlabeled leucine so that less than 100% of the label was incorporated into trichloroacetic acid precipitable material within a 5 minute pulse. Aliquots of the culture were removed at the desired times and shaken with 6 µM unlabeled leucine and ^{3}H-leucine (5 µCi/ml) for 5 minutes at 28°C. Under these conditions, a maximum of about 50% of the total label added was incorporated into protein. The labeled cells were then either extracted or treated with 5% trichloroacetic acid, as described below, to measure incorporation of label into luciferase or total protein during the pulse.

Measurement of Rates of Total Protein Synthesis and Turnover. The rate of protein synthesis was determined by measuring the incorporation of radioactivity (^{3}H-leucine) into trichloroacetic acid precipitable material following a 5 min pulse. Trichloroacetic acid precipitates were formed by vortexing 1.0 ml of culture with 0.1 ml of 50% (w/v) trichloroacetic acid in a 1.5 ml Eppendorf centrifuge tube. Incubation of these mixtures at room temperature for 1 to 24 hours made no difference in the final results. Precipitates were collected by centrifugation at 9000 x g for 4 minutes; the supernatant solutions were saved for counting. The precipitates were washed with 0.1 ml of 1% BSA in water and 1.0 ml of 5% trichloroacetic acid, and then with 1.0 ml of ice-cold absolute ethanol:anhydrous diethyl ether (1:1). Samples were air dried and dissolved in 0.1 ml 1 N NaOH with gentle heating. The base was neutralized with 0.1 ml 1 N HCl and diluted with 0.8 ml distilled water.

To count trichloroacetic acid supernatants or redissolved precipitates, 0.5 ml was dissolved in 5 ml of 3:1 (v/v) xylene:Triton X-114 with 0.4% PPO, and counted for 2 min in the ^{3}H + ^{14}C window of a Beckman LS-230 Liquid Scintillation System.

The rate of protein turnover was measured as the rate of release of radioactivity from the trichloroacetic acid insoluble material following a chase with nonradioactive leucine at 750 µg/ml. This method might be expected to give low numbers for turnover due to reutilization of the label. However, when we chased the label with 75 µg/ml cold leucine, we measured the same rate as for the 750 µg/ml cold leucine chase, showing that the measured rates are not too low as a result of reutilization of the radioactive leucine.

Assay and Purification of Luciferase. Luciferase activity *in vivo* and *in vitro* was measured using a photomultiplier-photometer similar to that described by Mitchell and Hastings (14). Activity *in vivo* was taken as the intensity of light emitted from one ml of culture in a scintillation vial.

Activity *in vitro* was determined using the standard flavin injection assay (15). Cell extracts were prepared by centrifuging a volume of culture for 6 minutes at 20,000 x g at room temperature, removing the medium from the cell pellet and resuspending the cells in the desired volume of 10 mM EDTA, 50 mM sodium-potassium phosphate, 1 mM dithioerythritol, pH 7.0 and freezing at -20°C to complete lysis. For preparation of antibodies, highly purified luciferase was prepared by the standard methods; an additional chromatography step using aminohexyl-Sepharose 4B was used to remove trace impurities (15,16).

Generation, Purification and Characterization of Antiluciferase IgG. New Zealand white female rabbits were injected intrascapularly with 1 ml of pure luciferase (1 mg/ml) in Freund's complete adjuvant. Secondary injections, identical to the primary injection except that Freund's incomplete adjuvant was used, were administered monthly and the rabbits were bled 2 weeks after each injection. Only secondary antibodies were used in these experiments.

The IgG fraction from immune rabbit serum was purified by a dextran sulfate fractionation followed by two ammonium sulfate precipitations, and DEAE-cellulose column chromatography. The pure IgG was dialyzed into 45 mM potassium phosphate buffer, pH 8.0, 150 mM NaCl, 0.005% sodium azide (phosphate-buffered saline) before use.

Titrations were performed to determine the ability of the purified IgG to neutralize the activity of the luciferase. Pure luciferase was accurately diluted to 60 to 70 µg/ml using phosphate-buffered saline, 0.05% BSA, 1 mM dithioerythritol (dilution buffer) and increasing amounts (0-3 µg) were added to a series of tubes each containing a constant amount (200-400 µg) of IgG. Four tubes were prepared at each luciferase concentration, two containing IgG and two control tubes containing an equal volume of phosphate-buffered saline. The total volume of each sample was adjusted to 200 µl with dilution buffer. The samples were incubated at room temperature for 4 hours and the luciferase activity of each determined by the standard flavin injection assay (15). It has been shown that the dithioerythritol present does not interfere with the inactivation reaction, and that the inactivation is complete after 3 hours (17). The activity remaining after 4 hours was plotted versus the initial activity added to each tube (determined by assay of the control tubes). The x-intercept of the linear portion of the resulting curve at high luciferase concentration was taken as the units of luciferase (and therefore the µg of pure luciferase) inactivated by the amount of IgG added. The IgG characterized in this manner was used to determine the specific activity of luciferase in crude lysates by the same method, except that the luciferase activity remaining after 4 hours was plotted versus the volume of lysate added. The x-intercept of this plot then gave directly the volume of lysate containing the amount of luciferase (antigen) inactivated by the IgG. The ratio of the units of luciferase activity in that volume to the µg luciferase antigen in that volume was taken as the specific activity of the luciferase in the lysate.

Direct precipitation of luciferase from crude cell lysates with IgG was done using lysates which had been centrifuged at 100,000 x g for 1 hour at 4°C, because use of uncentrifuged lysates or lysates centrifuged for 20 min at 18,000 x g resulted in large amounts of nonspecific precipitation. In a typical experiment, extracts of cells taken at various times throughout growth of a culture were prepared. The concentration of luciferase in the extract prepared at the peak of luminescence of the culture was estimated based on its luciferase activity measured *in vitro*. The volume of this extract that contained approximately 5µg of luciferase was determined and the same volume was taken from all other extracts made throughout the experiment. To each extract, enough IgG was added to inactivate 7.5 - 10 µg of pure luciferase (determined by the neutralization titration discussed above). The resulting mixtures were allowed to react at 5°C for 12 to 16 hours; the precipitate was collected by centrifugation at 9000 x g for 4 minutes. The precipitates were washed twice with 20 mM sodium-potassium phosphate buffer, pH 8.0. For scintillation counting, the precipitates were dissolved in 100 µl of 1 N NaOH with heating. The dissolved precipitates were then transferred into 5 ml of 0.4% PPO, 3:1 (v/v) xylene:Triton X-114 in a scintillation vial. To neutralize the base, 100 µl of 1 N HCl was added to each vial. Samples were counted for 2 minutes in the ^{3}H + ^{14}C window of a Beckman LS-230 Liquid Scintillation System.

Sodium Dodecyl Sulfate Gel Electrophoresis, Detection of Antigen On Gels and Fluorography. Proteins were resolved on 15% polyacrylamide slab gels in 0.1% $NaDodSO_4$ as described by Laemmli (18) and visualized by staining with Coomassie Brilliant Blue R-250. Antigenic material within the gels was detected by an immunoreplicate overlay technique (19). The gel slabs were overlaid with a 1:4 dilution of the antiserum with 0.8% agarose (Bio-Rad), so that the final concentration of agarose was 0.6%. The overlay was developed at 37°C for 6 hours. The agarose was then removed from the gel slab, washed, stained with Coomassie Brilliant Blue, and dried onto Whatman 3 MM paper. Less than 1 µg of antigen was readily detected by this method. Radioactive material in gels was detected by the quantitative fluorography technique described by Bonner and Laskey (20) and Laskey and Mills (21). Kodak X-Omat RP (RP-5) film was used and developed films were scanned using an Ortec Model 4310 Microdensitometer. By this method, 5000 cpm of tritium per cm^2 of gel gave a film optical density of 0.3 after 10 days exposure.

RESULTS

Characterization of Anti-luciferase IgG—Our first efforts were directed toward characterizing the anti-luciferase IgG that we had prepared by the methods described under "Experimental Procedures." To determine the specificity of the antibody, we examined the proteins precipitated by the IgG from a crude *V. harveyi* lysate (Fig. 1). The ability of the antibody to bind to and precipitate fragments of the luciferase was demonstrated by immunoreplicate electrophoresis (19) (Fig. 2). It is evident in Fig. 2 that, as reported (9), the α subunit (M_r = 42,000) was destroyed by the chymotrypsin while the β subunit (37,000) was not. The fragments from the α subunit, the γ family (M_r ~ 30,000) and the δ family (M_r = 11,000–15,000) (11), were clearly visible in the Coomassie blue-stained gel. All of the bands visible in the stained gel were also visible in the immune overlay except the smallest of the δ bands. It is not clear whether the 11,500-dalton fragment lacks the minimum of two determinants necessary to form a precipitating complex or whether it is so small that it was lost by diffusion, but it appears that by this technique it is possible to miss detection of some small fragments of luciferase.

The above results indicated that our antibody was specific for luciferase and did not precipitate other proteins from the centrifuged lysates and that it was directed against a sufficiently broad spectrum of determinants to allow complexation with a wide array of fragments of luciferase.

[1] The "Experimental Procedures" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document 81M-1402; cite authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

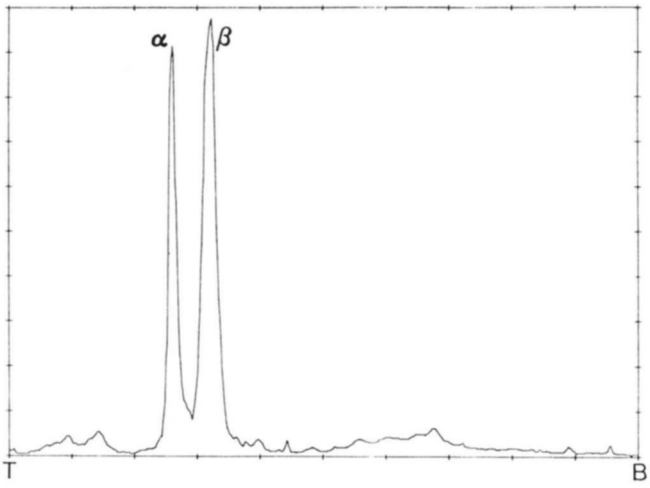


FIG. 1. **Quantitative fluorography of NaDodSO$_4$-polyacrylamide slab gel electrophoresis of antibody precipitate formed from a crude *V. harveyi* extract.** At a culture OD^{660} of 0.8, *V. harveyi* cells were labeled for 10 min with 2 μCi/ml of culture of [^{3}H] leucine. Five ml of the labeled cells was then harvested by centrifugation, lysed, and centrifuged at 100,000 × *g* for 1 h at 5 °C. To 0.3 ml of the supernatant, a 3.4-fold excess of anti-luciferase IgG over the amount of luciferase calculated to be present in the extract was added. This mixture was incubated for 16 h at 5 °C. The antibody precipitate was then collected by centrifugation and washed, dissolved in NaDodSO$_4$ gel sample buffer, and subjected to electrophoresis. The gel was treated for fluorography and exposed to film for 17 days. (The α and β bands were visible on the film after only 1 day, but we wished to view any minor contaminants.) The film was then scanned on a microdensitometer in the visible range. An identical precipitate was scintillation counted and found to contain about 77,000 dpm. The maximum OD of the α band on the densitometer scan was 1.6; that of the β band was 1.7. *T*, top of gel (cathode); *B*, bottom of gel (anode).

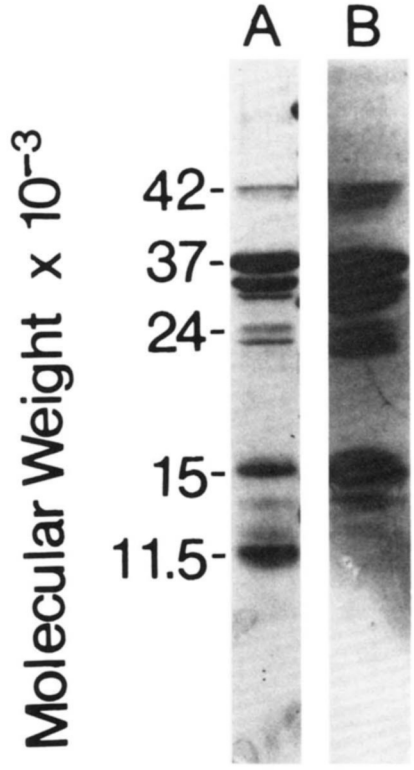


FIG. 2. **Use of immunoreplicate electrophoresis to determine specificity of anti-luciferase antibody for chymotryptic fragments of the enzyme.** The fragments of luciferase inactivated to 1% of its initial activity by treatment with chymotrypsin were separated by NaDodSO$_4$-polyacrylamide slab gel electrophoresis. Identical samples were run on the right and left sides of the same gel. One half of the gel was stained, and the other half was used to form an immune overlay. *Lane A*, gel of chymotryptic fragments; *Lane B*, immune overlay of lane identical with *Lane A*. The *numbers* at the *left* are approximate molecular weights for the fragments, determined by running molecular weight standards on the same gel.

Measurement of Total Protein and Luciferase Synthesis—We measured the specific rate of synthesis of luciferase and of total protein (trichloroacetic acid-insoluble counts) throughout growth of a culture (Fig. 3), as described under "Experimental Procedures." During the pulse, the cells took up less than 100% of the label added, so each point represents the rate of uptake of label into either total protein or luciferase at that time in growth. Furthermore, since OD^{660} was linearly related to cell number in the medium used, division by OD^{660} gives a number proportional to the rate of protein synthesis or of luciferase synthesis per cell.

Luciferase synthesis constituted about 7.5% of total protein synthesis shortly after induction, but the rate of luciferase synthesis per cell then declined steadily to reach zero in late log phase at about the time luciferase inactivation began. Although the rate of luciferase synthesis declined throughout exponential growth, the concentration of luciferase per cell increased throughout exponential growth to reach a maximum at an OD^{660} of about 1. The luciferase activity/ml of culture peaked somewhat later at an OD^{660} of about 1.5 (not shown in Fig. 3; see Fig. 6.). The rate of total protein synthesis per cell was maximal in mid-log phase and dropped to a low but detectable level in stationary phase.

Measurement of Anti-luciferase CRM[2]—The results of the experiment presented in Fig. 3 showed that luciferase synthesis had stopped before luciferase inactivation had begun, demonstrating that the observed rate of luciferase inactivation measured during late log phase and early stationary phase of

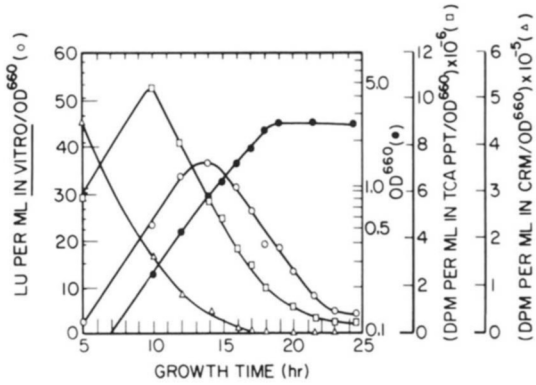


FIG. 3. **Total protein synthesis, luciferase synthesis, and luciferase activity per cell in *V. harveyi* cultures during growth.** 300-ml cultures of *V. harveyi* were grown in 1-liter baffled flasks at 27.5 °C in Bis-Tris minimal medium. At intervals during growth, a 6.4-ml aliquot of each culture was withdrawn and pulsed with 5 μCi/ml of [^{3}H]leucine and 6 μM cold leucine for 5 min. One ml of each aliquot was then used to form a trichloroacetic acid (*TCA*) precipitate, to monitor disintegrations/min incorporated into total protein. Five ml was harvested and resuspended in 1 ml of lysis buffer to form an extract, which was used to measure luciferase activity (LU/ml) and direct antibody-precipitable disintegrations/min. LU refers to arbitrary units of light intensity. ○, extractable luciferase activity per OD^{660}; □, disintegrations/min/OD^{660} incorporated into TCA-precipitable material in 5 min at the indicated growth time; △, disintegrations/min/OD^{660} incorporated into luciferase CRM in 5 min at the indicated growth time; ●, OD^{660} of culture.

[2] The abbreviations used are: CRM, immunologically cross-reacting material; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane; NaDodSO$_4$, sodium dodecyl sulfate; BSA, bovine serum albumin.

growth was the actual rate of inactivation, not the sum of inactivation and continuing synthesis. The experiment did not, however, address two very important questions. First, we did not know whether luciferase synthesized immediately following induction was stable throughout exponential phase growth or was degraded at some slow rate. Second, we knew nothing of the fate of the luciferase following inactivation in late log phase and early stationary phase of growth.

To answer these questions, cellular proteins were pulse-labeled with [^{3}H]leucine during early exponential phase and the fate of the labeled anti-luciferase CRM was determined by direct immunoprecipitation (Fig. 4). The luciferase CRM synthesized during early exponential phase of growth remained fully cross-reactive throughout the period of luciferase synthesis. The amount of CRM then decreased during stationary phase in concert with the amount of extractable luciferase activity. Fluorographs of duplicates of the antibody precipitates made in this experiment following gel electrophoresis (not shown) showed only two bands which co-migrated with the α and β subunits of the luciferase; these two bands appeared to be lost at the same rate.

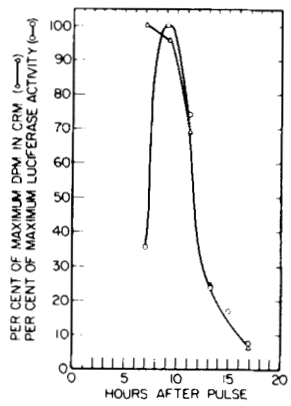
One very important aspect of this experiment is that the antibody precipitates were prepared using the supernatants of cell lysates centrifuged at 100,000 × *g* for 1 h. Therefore, any CRM present in the 100,000 × *g* pellets would not be detected.

Effects of Chloramphenicol and of Cyanide and Fluoride on Total Protein Turnover and Luciferase Inactivation—In our earlier publication (8), we reported that in a complex medium the inhibitors chloramphenicol, and cyanide and fluoride together, if added before the onset of luciferase inactivation, would prevent inactivation from occurring. However, if the inhibitors were added after the onset of luciferase inactivation, the inactivation continued for about 2 h before stopping. The levels of cyanide employed caused a rapid drop in ATP to about 25% of the level in exponential phase cells, while chloramphenicol caused a rapid increase in ATP to about twice the level in exponential phase cells.

To better understand the effects of these inhibitors on luciferase inactivation, we determined their effects on protein synthesis (Fig. 5). Chloramphenicol was found to effectively block protein synthesis within 10 min at 100 μg/ml culture. Addition of KCN to 1 mM, on the other hand, had only inhibited protein synthesis by 75% after 1 h while 1 mM KCN and 1 mM NaF had blocked 90% of protein synthesis after 1 h.

The effects of these inhibitors on total protein turnover in comparison with luciferase inactivation were determined by measuring the release of radioactive material into the trichloroacetic acid supernatant (Figs. 6 and 7). Fig. 6 shows that total protein turnover during exponential phase growth proceeded at a constant rate of about 0.7%/h. In early stationary phase, when the luciferase activity peaked and luciferase inactivation began, the rate of release of radioactivity from the trichloroacetic acid-insoluble fraction increased to about 1.7%h. However, chloramphenicol added at this point decreased the rate to about 0.4%/h after a lag of about 1.5 h. The chloramphenicol also caused a cessation in luciferase inactivation after about 1.5 h. In the experiment shown (Fig. 6), the luciferase inactivation stopped completely. However, in most other experiments (not shown), the effect of chloramphenicol was to slow the inactivation of luciferase, not to stop inactivation completely. The experimental cause of this variability is unknown, but in no other experiment was the inhibitor added precisely at the peak of extractable luciferase activity.

The experiment of Fig. 6 was repeated using KCN and NaF as the inhibitors (Fig. 7); the data prior to the peak of extractable luciferase activity were similar to those shown in Fig. 6, and only the latter part of the growth curve is shown. These data show that addition of KCN and NaF to the culture during stationary phase resulted in an immediate cessation of bulk protein turnover but did not stop luciferase inactivation until about 2 h after the addition. About 2.5 h after the inhibitors were added, there was a resumption of bulk protein turnover at a slow rate of about 0.4%/h.



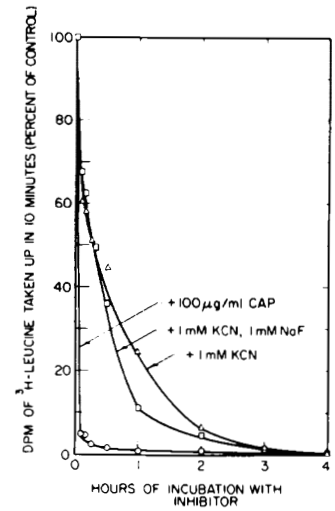


FIG. 4 (*left*). **Loss of luciferase CRM and luciferase activity in a *V. harveyi* culture.** A 330-ml culture of *V. harveyi* cells in a 1-liter baffled flask was grown at 27.5 °C in Bis-Tris minimal medium. When the culture reached an OD^{660} of about 0.2, two 108-ml aliquots were removed and placed in separate 1-liter flasks. 2 μCi/ml of [^{3}H] leucine was added to one culture. After 20 min of continued shaking, both cultures were centrifuged, and the labeled cells were washed once with 100 ml of fresh medium containing cold leucine and resuspended in the medium from the unlabeled culture, supplemented with cold leucine. At intervals following the pulse, extracts of the culture were prepared using 10 ml of culture for each ml of extract. After centrifugation at 100,000 × *g* for 1 h at 5 °C, the luciferase activity in the extracts was measured. The luciferase CRM in these extracts was also determined as direct antibody-precipitable disintegrations/min. Both of these values are represented as the per cent of the total present in the peak extract. The maximum present in the CRM at the peak (set equal to 100%) was 14,000 dpm. ○, extractable luciferase activity; △, CRM.

FIG. 5 (*right*). **Effects of KCN, of KCN and NaF, and of chloramphenicol on total protein synthesis in *V. harveyi*.** A culture grown in Bis-Tris minimal medium was subdivided into 4 cultures, 1 control and 3 with inhibitors. Aliquots (2 ml) were removed at various times following addition of the inhibitors and shaken in 20-ml scintillation vials with 2 μCi of [^{3}H]leucine for 10 min. Then, 1 ml of each aliquot was pipetted into 0.1 ml of 50% trichloroacetic acid (*TCA*), and the TCA-precipitable material was treated and counted as described under "Experimental Procedures." The amount of [^{3}H]leucine incorporated into TCA-precipitable material in 10 min after varying periods of incubation with inhibitor(s) is expressed as the per cent of the disintegrations/min incorporated in 10 min by the control culture at the same time. ○, culture containing 100 μg/ml of chloramphenicol (*CAP*); △, culture containing 1 mM KCN; □, culture containing 1 mM KCN and 1 mM NaF.

This result suggested that luciferase inactivation could continue without protein degradation, raising the possibility that under these conditions, inactive luciferase might accumulate and be detected as CRM with the anti-luciferase antibody. To test this possibility, we repeated the experiment shown in Fig. 4, but added either chloramphenicol or NaF and KCN to the cultures (Table I). In all experiments, the cross-reactive material in the 100,000 × *g* supernatant was lost in parallel with the activity; loss of luciferase protein was not blocked under conditions which blocked total protein turnover.

Activity Neutralization in Uncentrifuged Lysates—As

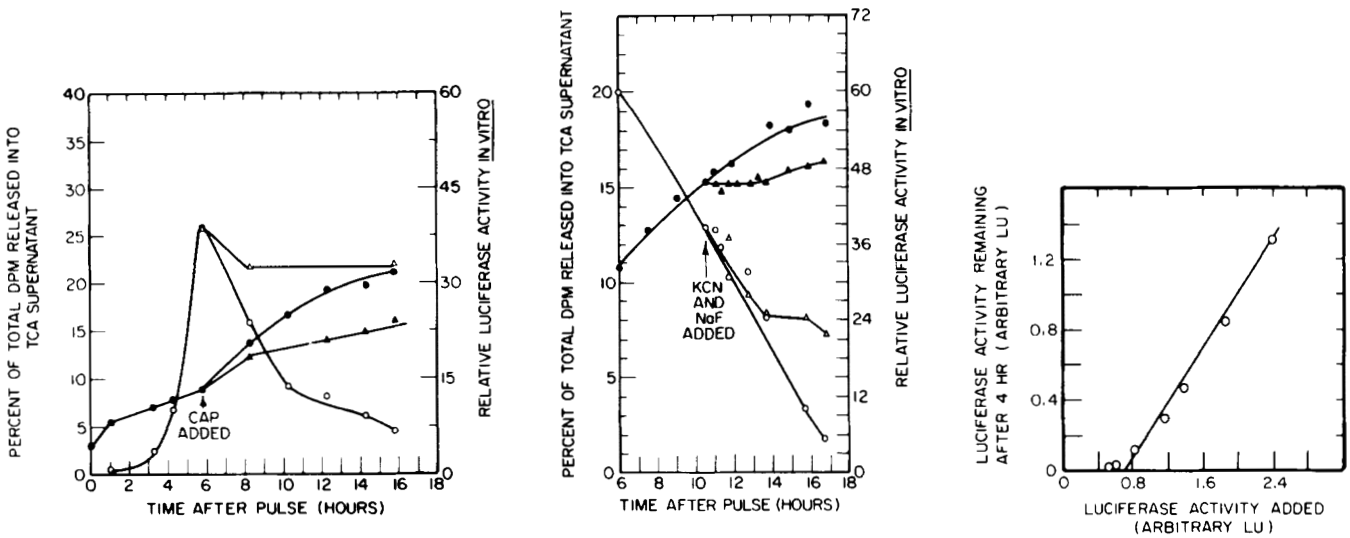


FIG. 6 (*left*). **Bulk protein turnover in *V. harveyi*: effect of chloramphenicol.** A 300-ml Bis-Tris minimal culture of *V. harveyi* cells in a 1-liter baffled flask was grown at 27.5 °C, to an OD^{660} of about 0.24. At this point, two 90-ml aliquots were removed and placed in 300-ml baffled flasks. One culture was pulsed for 20 min with 1 μCi/ml of [^{3}H]leucine. The labeled cells were harvested by centrifugation and washed twice with 70 ml of fresh medium plus 750 μg/ml of cold leucine. These labeled cells were then resuspended in medium from the parallel unlabeled culture, to which 750 μg/ml of cold leucine had been added. After 30 min of growth, this labeled culture was split into two 40-ml cultures in 300-ml baffled flasks. To one of these, 100 μg of chloramphenicol (*CAP*)/ml of culture was added at the peak of luciferase activity. The other culture was used as the control. Trichloroacetic acid (*TCA*) precipitates were formed from 1-ml aliquots of both cultures at various times following the pulse, and the luciferase activities *in vitro* of both cultures were also monitored. Bulk protein turnover is expressed as the per cent of the total disintegrations/min initially incorporated into TCA-precipitable material which is released into the TCA supernatant with time. ○, control culture luciferase activities *in vitro*/ml of culture; △, luciferase activities *in vitro*/ml of chloramphenicol-containing culture; ●, per cent of TCA-precipitable counts released in control culture; ▲, per cent of TCA-precipitable counts released in culture to which chloramphenicol was added.

FIG. 7 (*center*). **Bulk protein turnover in *V. harveyi*: effect of KCN and NaF.** This experiment was performed in a manner identical with that shown in Fig. 6, except that the culture was pulsed at an OD^{660} of about 0.16. The inhibitors (1 mM KCN and 1 mM NaF, added from stock 1 M solutions in water) were added to the culture about 4 h after peak luminescence was reached, rather than at the peak. The results show that portion of the growth after peak luminescence was reached. ○, control culture luciferase activities *in vitro*/ml of culture; △, luciferase activities *in vitro* of cultures containing 1 mM KCN and 1 mM NaF; ●, per cent of trichloroacetic acid (*TCA*)-precipitable counts released in control culture; ▲, per cent of TCA-precipitable counts released in culture to which 1 mM KCN and 1 mM NaF were added.

FIG. 8 (*right*). **Luciferase activity neutralization assay.** The activity titration was performed as described under "Experimental Procedures" using 200 μg of pure IgG and increasing amounts of pure luciferase. The *x-intercept* indicated that the IgG inactivated 0.78 μg of luciferase. The correlation coefficient of the line was greater than 0.99. LU refers to arbitrary units of light intensity.

mentioned above, all direct antibody precipitates were made using lysates that had been centrifuged at 100,000 × *g* for 1 h immediately before use. There was, therefore, a possibility that the luciferase was not being degraded to fragments too small to be precipitated by the antibody but rather was being converted to an insoluble and inactive form that was removed by the 100,000 × *g* centrifugation. To test this hypothesis, the specific activity of the luciferase in crude extracts was determined by the luciferase activity neutralization titration described under "Experimental Procedures" (see Fig. 8).

The luciferase in lysates of cells taken at peak luminescence had the same specific activity as the luciferase in lysates of cells taken in late stationary phase, if the lysates were centrifuged prior to the titration (Table II). This finding was consistent with the failure to detect excess (inactive) luciferase CRM in late stationary phase extracts using direct antibody precipitation. However, if the lysates were not centrifuged prior to the titration, there was a dramatic decrease in the specific activity of the luciferase in late stationary phase lysates relative to lysates prepared at peak luminescence. This result suggests that the inactive luciferase (CRM) in late stationary phase cells is present in the 100,000 × *g* pellet of cell lysates and is capable of binding to luciferase-inactivating antibodies. An additional finding from this experiment was that the specific activity of the luciferase in uncentrifuged lysates taken at the peak of luminescence was only 35% of the specific activity in the same lysate following centrifugation. This result may be due to nonspecific binding, but it could indicate that inactivation of luciferase begins prior to peak luminescence.

The activity neutralization method relies on antibody molecules binding to and inactivating luciferase. The inactivating antibodies are probably a small subpopulation of the total collection of anti-luciferase antibodies which recognize only a limited number of determinants. It appeared likely that after inactivation some of the determinants present in active luciferase might be lost so that the total amount of CRM estimated by this technique would be low. To test this hypothesis, we treated pure luciferase with chymotrypsin until about 1% of the initial activity remained. We then used the activity neutralization assay to determine the specific activity of the protease-treated sample (Table II). The result of the titration showed that even though 99% of the luciferase molecules had been inactivated by the action of chymotrypsin, 93% of the luciferase molecules were still recognized by the luciferase-inactivating antibodies.

DISCUSSION

There are three means by which cells can control the amount of a particular enzymatic activity *in vivo*: 1) production of the enzyme, either by *de novo* synthesis or zymogen activation; 2) modulation of enzyme activity by reversible ligand binding or covalent modification; and 3) irreversible inactivation of the enzyme, either by physical modification (*e.g.* denaturation), covalent modification, or proteolytic degradation. While much study has focused on the first two

TABLE I

Loss of luciferase CRM and luciferase activity in V. harveyi cultures in the presence and absence of KCN and NaF or of chloramphenicol

In the two experiments represented here, *V. harveyi* cultures were pulse-labeled during early log phase growth using [^{3}H]leucine. In both experiments, the original culture volume was 330 ml in Bis-Tris minimal medium in a 1-liter flask, and the growth temperature was 28 °C. In Experiment 1, 160 ml of the culture was labeled with 3 μCi/ml of [^{3}H]leucine for 20 min at an OD^{660} of 0.4. In Experiment 2, 100 ml of the culture was labeled similarly, but at an OD^{660} of 0.1. In both experiments, shortly after peak extractable luciferase activity was attained, the culture was split into three equal subcultures (35 ml each in Experiment 1 and 21 ml each in Experiment 2) in 300-ml flasks. KCN and NaF were added (from stock 1 M solutions in water) to one subculture, to make it 1 mM in each. 100 μg/ml of chloramphenicol was added (as the solid) to another subculture. The third subculture was used as the control. Luciferase activity present in extracts of these cultures prepared at various times following addition of the inhibitors was measured. Luciferase CRM in these extracts was also determined as direct antibody-precipitable disintegrations/min. Results are expressed as the per cent of the total present in the peak extract. In Experiment 1, the CRM in the peak extract contained 17,600 dpm. In Experiment 2, it contained 25,500 dpm. Details of all methods are given under "Experimental Procedures."

Experiment	Addition to culture	*Hours* after addition	% of maximal luciferase activity remaining	% of maximal CRM remaining
1	None (control)	0	64	60
		2.75	32	34
		5.75	17	15
	1 mM KCN and 1 mM NaF	1.25	60	66
		4.25	56	62
		5.75	40	37
	100 μg/ml of chloramphenicol	1.25	64	68
		2.75	64	64
		4.25	44	49
2	None (control	0	48	46
	1 mM KCN and 1 mM NaF	5.5	28	27
	100 μg/ml of chloramphenicol	5.5	32	29

TABLE II

Luciferase activity neutralization titrations of crude cell extracts and proteolyzed luciferase

Sample titrated	Relative specific activity determined[a]		
	Whole extract[b]	100,000 × *g* supernatant[b]	Pure luciferase[c]
V. harveyi extract[d] (peak luminescence)	0.35	1.0	
V. harveyi extract[d] (late stationary phase)	0.10	0.97	
V. harveyi luciferase (native, pure)			1.0
V. harveyi luciferase treated with chymotrypsin, 1% activity remaining[e]			0.07

[a] The specific activity of the luciferase CRM present in a given sample was calculated by assuming that the amount of IgG used would inactivate 0.78 μg of luciferase (C. A. Reeve (1980) Ph.D. thesis, University of Illinois, Urbana, IL).

[b] Specific activities measured were divided by the specific activity of the luciferase CRM in the 100,000 × *g* supernatant of the peak extract to give the relative specific activities reported in the table.

[c] Specific activities measured were divided by the specific activity of the native pure luciferase to give the relative specific activities reported in the table.

[d] 300-ml cultures of *V. harveyi* cells were grown in sodium chloride complete medium in 1-liter baffled flasks at 28 °C. At peak luminescence (8 h), 80 ml of the culture was harvested; the cells were lysed using 4 ml of 10 mM EDTA, 50 mM sodium-potassium phosphate, pH 7.0, 1 mM dithioerythritol, and frozen. In late stationary phase, when the extractable luciferase activity had decreased to 0.4 times its peak value, 80 ml of the culture was similarly lysed in 2 ml of the same buffer. After thawing, half of each lysate was centrifuged for 1 h at 100,000 × *g*, at 5 °C. The other half was left alone. Both whole extracts and 100,000 × *g* supernatants were then assayed and diluted using 45 mM potassium phosphate, pH 8.0, 150 mM NaCl, 0.005% sodium azide, 0.05% BSA, 1 mM dithioerythritol, so that 25 μl of extract would contain 10 to 15 light units of activity. Activity neutralization titrations were then carried out using anti-luciferase antibody, as described under "Experimental Procedures."

[e] Pure *V. harveyi* luciferase was inactivated with chymotrypsin as described (10).

processes, it is only recently that the importance of selective inactivation of enzymes has been appreciated.

In recent years, a considerable body of experimental evidence concerning the occurrence of selective enzyme inactivation and turnover in microbes has been accumulated (22–24). In this context, "inactivation" generally refers to the irreversible loss *in vivo* of enzymatic activity, while "turnover" refers to the cleavage of the protein to the constituent amino acids (22). Operationally, turnover is generally indicated by loss of cross-reacting material. There are presently numerous reports in the literature of selective inactivation, often by as yet unknown mechanisms, of microbial enzymes under specific physiological conditions. These reports, the known mechanisms of enzyme inactivation, and the possible roles of enzyme inactivation in the physiology of microbes have been reviewed by Switzer (22).

We have shown that, during stationary phase, the luciferase protein is lost from centrifuged lysates at the same rate as luciferase activity, about 10 times the rate of total protein turnover. There are two explanations for this observation that are not mutually exclusive. Either the luciferase protein is being degraded to fragments or otherwise modified so that the anti-luciferase antibody does not recognize it or form a precipitating complex, or the luciferase protein is being inactivated to form an insoluble precipitate that is removed by the centrifugation step. Prouty *et al.* (25) have reported that aberrant proteins produced either by incorporation of amino acid analogs or by treatment with puromycin precipitate to form electron-dense inclusions within *Escherichia coli* cells and that the rapid turnover of the proteins is associated with removal of the inclusions. They suggested that protein turnover in these cells proceeds by a denaturation step followed by proteolysis. This explanation appears to accommodate their observations, and on reflection, it could be incorporated into virtually any hypothesis regarding the inactivation and turnover of specific enzymes. For example, the inactivation of glutamine phosphoribosylpyrophosphate amidotransferase *in vivo* in *Bacillus subtilis* appears to be an oxidation-mediated process (26–28). One consequence of this oxidation *in vitro* is decreased solubility of the protein, so it is not unlikely that turnover of the protein *in vivo* proceeds via the pathway oxidation-precipitation-turnover. In fact, to prove for any specific turnover that degradation is not preceded by precipitation could well be exceedingly difficult. One would have to demonstrate turnover *in vitro* in a particle-free extract to rule out the possibility that turnover occurs from insoluble protein aggregates.

The results of our experiments suggest that the inactivation of bacterial luciferase is associated with a decrease in solubility of the enzyme. This postulate is consistent with the loss of cross-reacting material from the soluble fraction of the cell lysates at the same rate as luciferase activity and with the presence of excess cross-reacting material in the uncentrifuged

cell lysates, giving a lower specific activity for the luciferase (light units/unit of cross-reacting material) in late stationary phase cells.

Total protein turnover in *V. harveyi* appeared to be similar to that in *E. coli* (23, 24) but to proceed at a slower rate, possibly due to the lower growth temperature. The rate of total protein turnover was about 0.7%/h during exponential phase and about 1.7%/h during stationary phase. Inhibition of protein synthesis with chloramphenicol was very rapid and effective, but the effect of chloramphenicol on protein turnover was delayed and incomplete. The effect of chloramphenicol on luciferase inactivation was about the same as on total protein turnover, suggesting some coupling between total protein turnover and luciferase inactivation. On the other hand, cyanide and fluoride, which were slow and ineffective in inhibiting protein synthesis, were very rapid and effective in blocking total protein turnover. However, their effect on luciferase inactivation was delayed by several hours. During the time following addition of cyanide that luciferase inactivation continued, the luciferase cross-reacting material continued to disappear from the soluble fraction of the cell lysate at the same rate as the luciferase activity. These findings demonstrate that the inactivation of luciferase can proceed in the absence of measurable protein turnover. We have not ruled out the possibility that under these conditions fragments of the luciferase are formed which are not precipitated by the antibody, yet are trichloroacetic acid-precipitable (*e.g.* peptides of M_r = 5,000–10,000). However, these findings are again consistent with a model in which the inactivation of luciferase is intimately associated with a dramatic decrease in solubility; under normal conditions, this inactivation may be followed by proteolytic degradation of the protein. It is possible that the inactivation-turnover of other enzymes occurs by the same general pathway, but for other proteins, demonstration of the insoluble protein as an intermediate between the active enzyme and free amino acids might be technically very difficult. With the bacterial luciferase system, however, the luciferase comprises about 5% of the soluble cell protein in fully induced cells in complex media, thereby facilitating demonstration of possible intermediate species.

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