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Biophysics

Isolation of the *in vivo* emitter in bacterial bioluminescence

(emission spectra/blue fluorescence protein)

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ABSTRACT **A blue fluorescence protein has been isolated and purified from extracts of the luminous bacterium *Photobacterium phosphoreum*. It is a single polypeptide of molecular weight 22,000 with absorption maxima at 274 and 418 nm. It is efficiently fluorescent (ϕ_F 0.45), with a fully corrected spectral maximum (476 nm) and distribution identical to the *in vivo* bioluminescence from this same type of bacterium. At low concentration this fluorescence shifts towards the red and becomes identical to the *in vitro* bioluminescence emission. This spectral shift apparently results from a change in the protein pulled by dissociation of the chromophore ($K_d \simeq 10^{-7}$ M). If the blue fluorescence protein is included in the *in vitro* bioluminescence reaction with reduced FMN, oxygen, aldehyde, and luciferase (*P. phosphoreum*), the bioluminescence spectrum is shifted towards the blue from its maximum at 490 nm to one at 476 nm, where it is again identical in all respects to the *in vivo* bioluminescence spectrum. This is accompanied by an increase in the initial light intensity by an order of magnitude at saturating levels of blue fluorescence protein, and the specific light yield of the luciferase is increased 4-fold. It is suggested that the blue fluorescence protein acts as a sensitizer of the bacterial bioluminescence reaction.**

In 1953 Strehler (1) reported a stimulatory effect of NAD^+ and NADH on the dim light emission of cell-free extracts of bioluminescent bacteria. Since this demonstration of the *in vitro* reaction of bacterial bioluminescence, the question of what compound actually emits the light has been an intriguing one. Although FMN is the only fluorescent product of the light reaction, it has been pointed out repeatedly that FMN cannot be the emitter due to the clear spectral difference between its fluorescence and the bioluminescence (2, 3). Several proposals for the emitting species, without making a distinction between the situations *in vivo* and *in vitro*, have been made during the last two decades. After an observation of the chemiluminescence of indoles, it was suggested that the bacterial emitter could be an indole moiety attached to luciferase (4, 5). In 1962 Terpstra (6) reported a substance isolated from extracts of *Photobacterium phosphoreum* that had a broad fluorescence in the 450-nm region and enhanced the bioluminescence activity of luciferase in the *in vitro* reaction. Later she reported (7) that the addition of $FMNH_2$ to a luciferase preparation results in the formation of a compound that is transformed by irradiation with UV light (366 nm) into a substance having a fluorescence maximum at 470 nm. She suggested that the first compound was a precursor of the light-emitting molecule in bacterial bioluminescence.

The first reports of the absorption spectrum of a solution of crystalline luciferase from *Photobacterium fischeri* showed a shoulder at 415 nm (8) or at 400 nm (9). While contamination by cytochrome is responsible for part of this (10), Cormier and Kuwabara (11) showed that excitation of their luciferase at 420 nm resulted in a fluorescence with a spectral maximum at 515 nm, and further, that on the addition of the proper amount of hydrosulfite, this fluorescence shifts towards the blue so that the maximum closely approaches that of the bioluminescence. Eley *et al.* (12) had a chromophore associated with their crystalline luciferase preparations that also had a fluorescence maximum at 490 nm and an excitation maximum at 390 nm. In 1969 Cormier *et al.* (13) proposed an NAD^+-aldehyde adduct as the emitter. Model compounds have an absorption at 420 nm and an emission at 515 nm, consistent with the earlier observation made on crystalline luciferase (11).

More recently, after Mitchell and Hastings (14) claimed that the emitter must be some sort of flavin-derived species (2), three proposals for its structure were put forward, supported almost solely by the similarity of the fluorescence of model compounds to the bioluminescence spectra. Eley *et al.* (12) proposed the cation of FMN, McCapra and Hysert (15) a quinoxaline which could be formed as a transient product on opening of the ring of the FMN molecule, and Balny and Hastings (16) together with Tu and Hastings (17) proposed an $FMNH_2$ molecule substituted in the 4a-position.

It had been tacitly assumed that the mechanism of reaction and identity of the emitter are the same *in vivo* as *in vitro*. The possibility of a difference has been raised by the recent discovery of a bacterial type emitting at 545 nm (18). In this paper we show that a protein-bound chromophore can be isolated from extracts of the bioluminescent bacteria *P. phosphoreum* that is closely associated with luciferase and that fulfills all the conditions to qualify it as the *in vivo* emitter.

MATERIALS AND METHODS

The bacterium *Beneckea harveyi*, strain 392 in the classification scheme of Reichelt and Baumann (19), previously designated "MAV," was obtained from J. W. Hastings (Harvard University). The type "A-13" was isolated from the light organ of the "silver macrourid" fish by J. Paxton (Australian Museum) and has been identified as *Photobacterium phosphoreum* (J. Fitzgerald, private communication). The type *Photobacterium fischeri*, strain 399, was obtained from F. H. Johnson (Princeton University). The bacteria were grown and the luciferase and FMN were purified as described (20, 21). The blue fluorescence protein was routinely assayed by its fluorescence intensity at 470 nm when excited at 420 nm, with an Aminco-Bowman Spectrofluorimeter. Luciferase activity was determined with a digital photometer, designed and constructed by G. J. Faini, which was calibrated for absolute photon sensitivity with the luminol chemiluminescence reaction as a light standard (22). This standard is directly traceable to the National Bureau of Standards (NBS) Lamp (23) and its calibration has been confirmed by three independent methods (24–26). NADH dehydrogenase was purified from *P. fischeri* 399 by C. White and coupled efficiently with all types of luciferases used. All other chemicals were of the best commercial grades.

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Table 1. Enhancement of luciferase activity and total light by the blue fluorescence protein*

Blue fluorescence protein, μM	Initial light intensity, 10^{11} photons s^{-1}	Light intensity decay rate, s^{-1}	Total light, 10^{11} photons
0	1.8	0.13	13.8
12.6	2.7	0.15	18.0
30	5.0	0.18	27.8
56	5.7	0.17	33.5
101	9.3	0.22	42.3
181	11.2	0.25	44.8
106†	0.002	0.10	0.02
BSA‡	2.7	0.15	17.7

The reaction mixture contained (final concentrations) 50 mM phosphate, 0.1 mg of luciferase (type A13) per ml, and blue fluorescence protein. The bioluminescence was initiated by adding 10 μl of a saturated solution of dodecanal in methanol, followed by rapid addition of 0.2 ml of an 80 μM $FMNH_2$ solution. Final volume was 0.45 ml; temperature 23°; pH 7.0.

* All results are an average of four to six observations and have a coefficient of variation of ±10%.

† No luciferase.

‡ Bovine serum albumin (BSA) (95 μM) (6.5 mg/ml) substituted for blue fluorescence protein.

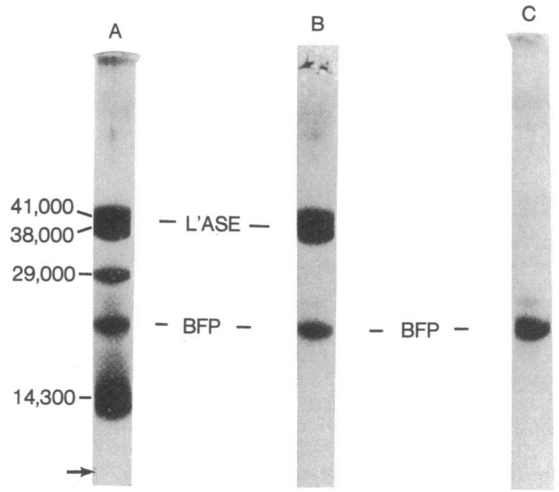

FIG. 1. Molecular weight and homogeneity of blue fluorescence protein (BFP) by sodium dodecyl sulfate/acrylamide (10%) gel electrophoresis by the method of Weber and Osborn (29). (*A*) Blue fluorescence protein (15 μg) with the markers *P. fischeri* luciferase (L'ASE, 20 μg), carbonic anhydrase, and lysozyme; the arrow indicates the dye front. (*B*) Luciferase and blue fluorescence protein (15 μg). (*C*) Blue fluorescence protein (30 μg).

Absorption spectra were taken with a Cary 14 spectrophotometer. Absolute fluorescence and bioluminescence spectra were obtained with an on-line computer-spectrofluorimeter system (27) at a band-width of 5 nm. The sample at room temperature (23°) was contained in a cuvette with a path-length of only 1 mm in the emission direction to minimize corrections necessary for self-absorption. Spectra were also corrected for the spectral sensitivity of the photomultiplier-monochromator system by reference to an NBS Standard of Spectral Radiance. The *in vitro* bioluminescence emission spectra were all determined in a reaction mixture containing 50 mM potassium phosphate (pH 7), 0.7 mg of bovine serum albumin per ml, 215 μM NADH, and 3.2 μM FMN in a total volume of 1.5 ml at 23°. To this was added 10 μl of a saturated solution of dodecanal in methanol, 50 μl of NADH dehydrogenase (A_{280} 3.14, specific activity 1.2 μmol of NADH min^{-1} mg^{-1}), and luciferase of the type under study in an amount that would give an initial flash height of 10^{12} photons-s^{-1} if assayed using dodecanal by the normal procedure of injection of $FMNH_2$ (20, 21).

For the reactions reported in Table 1, optical path lengths of both 5 mm and 1 mm were used. The highest concentrations of blue fluorescence protein used required a correction for self-absorption and re-emission of fluorescence of only 1.3.

Fluorescence lifetimes were determined for the fluorescence at 470 nm and at 490 nm by a single photon counting technique, using an air-gap spark source with most of the excitation at 358 nm, since the sample was contained in a glass cuvette (28).

RESULTS

From extracts of the bacterium type A-13 a "blue fluorescence protein" was isolated. Its association with the luciferase provided a convenient method of purification, by carrying it along through the several stages of luciferase purification detailed elsewhere (20, 21). Minor modifications were made: ammonium sulfate precipitation from the cell lysate, desalting on Sephadex G-75, adsorption to DEAE-cellulose (50 mM phosphate, pH 7.6) and elution with 0.15 M phosphate (pH 7.6), and adsorption on DEAE-Sephadex (A-50, 50 mM, pH 7.6) with elution by a phosphate gradient (0.05–0.35 M, pH 7.6). Some blue fluorescence protein separates from the luciferase at the Sephadex G-75 and A50 stages but the two fractions can be recombined. After the A50 step the mixture was subjected to a slow molecular sieving (Sephadex G-75 superfine, 5 × 100 cm). The luciferase eluted in the column front (molecular weight ~80,000) and the blue fluorescence protein was retarded, consistent with its much lower molecular weight. At this point it had a homogeneity of about 70% and was further purified by repeated gel filtration (Sephadex G-75 superfine, 3 × 85 cm). From 500 g of cell paste the final yield of blue fluorescence protein was about 10 mg. The relative fluorescence 420 → 470 nm was improved about 30 times from the first Sephadex step, based on absorbance at 275 nm.

The homogeneity of the blue fluorescence protein was determined by sodium dodecyl sulfate gel electrophoresis. In Fig. 1*C* an impurity can be seen just above the heavily staining protein band; it is estimated to be about 10% of the total protein. A 90% purity in this preparation is also supported by the absorption spectrum data. Fig. 1*B* shows the clear difference between the blue fluorescence protein and luciferase, which is a doublet of two nonidentical subunits (30), not resolved in this photograph. Luciferase was used as a marker, along with carbonic anhydrase and lysozyme, to determine the molecular weight of the blue fluorescence protein (Fig. 1*A*) (29).

The molecular weight of blue fluorescence protein is 22,000, an average of the results from sodium dodecyl sulfate gel electrophoresis, sedimentation equilibrium monitored at 270 and 420 nm, and sedimentation velocity (420 nm). Calibrated gel filtration (Sephadex G-75 superfine) also gave a result consistent with this molecular weight. Since the same molecular weight was obtained by sedimentation and sodium dodecyl sulfate gel electrophoresis, blue fluorescence protein has a single polypeptide chain.

On alkaline disc gel electrophoresis (31) the blue fluorescence protein undergoes denaturation and aggregation. Before staining, however, only one fluorescent band was evident and it corresponded in R_F to the heaviest staining protein band.

Fig. 2 is the absorption spectrum of blue fluorescence protein. The protein concentration was determined by the dye-binding method of Bradford (32), and the extinction of the chromophore at 418 nm was about 4000 M^{-1} cm^{-1}, calculated from the flu-

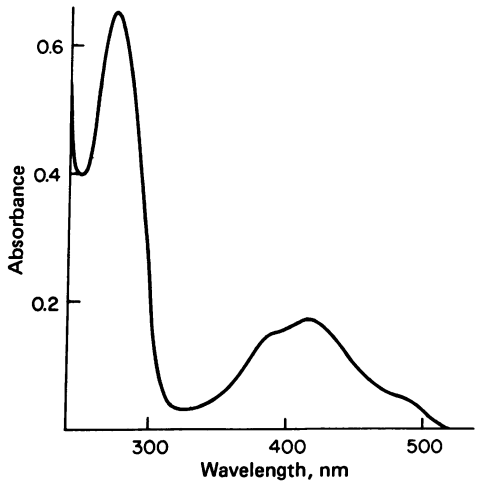

FIG. 2. Absorption spectrum of the blue fluorescence protein (1 mg/ml) in 50 mM phosphate, pH 7.0, at 23°.

orescence yield and lifetime. The mole ratio of the chromophore to protein is therefore 0.9:1.0, consistent with the estimated purity of the preparation and an assumed 1:1 ratio of chromophore to protein.

The fluorescence of the blue fluorescence protein is shown in Fig. 3*A*. This fluorescence is identical to the *in vivo* bioluminescence spectrum from the same type of bacterium (Fig. 3*B*). Both these spectra are similar, if not identical, to the bioluminescence spectrum of *P. phosphoreum*, first published by Spruit-van der Burg (33). Of all the emitters proposed and listed in the Introduction, none has the skewness, the small width at half-height, and the peak position exhibited by the *in vivo* emission of *P. phosphoreum* (33, 34).

Although the spectral matching shown in Fig. 3 alone makes this newly isolated blue fluorescence protein a good candidate for the bioluminescence emitter *in vivo*, a more striking property is the effect of including it in the *in vitro* reaction mixture—the *in vitro* spectrum shifts towards the blue to become an *exact match* for the *in vivo* spectrum. This is demonstrated in Fig. 4*A* (without blue fluorescence protein) and Fig. 4*B* (with the protein, ~70 μM); and Fig. 4*B* is identical to Fig. 3*B*. Different types of bacteria have *in vivo* bioluminescence maxima ranging from 472 to 505 nm, and recently one has been isolated at 545 nm (18), but the maxima of the *in vitro* spectra all cluster around 496 nm (18, 35). Efforts to shift the *in vitro* maximum by changes in external conditions, such as pH, addition of metal ions, temperature, acid denaturation, and the chain length of the aldehyde, have all failed (35). We observe that the *in vivo* spectra are similarly unaffected. The fact that the shift induced by blue fluorescence protein results in a spectral distribution that exactly matches the *in vivo* bioluminescence favors the idea that the blue fluorescence protein is itself the emitter under these conditions.

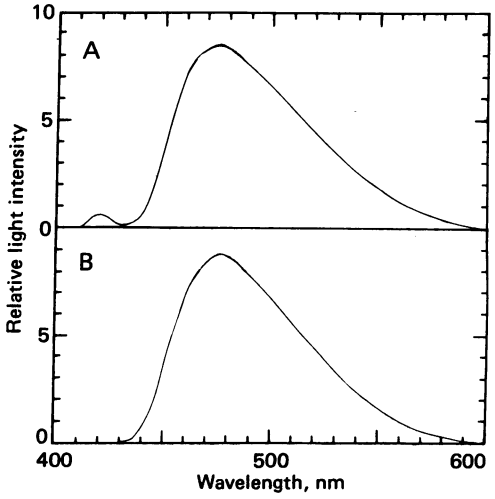

FIG. 3. (*A*) Fluorescence of the blue fluorescence protein (12 μM). Excitation at 420 nm, in 50 mM phosphate, pH 7.0, at 23°. (*B*) *In vivo* bioluminescence of *P. phosphoreum*, type A13. Experimental conditions are described in the text.

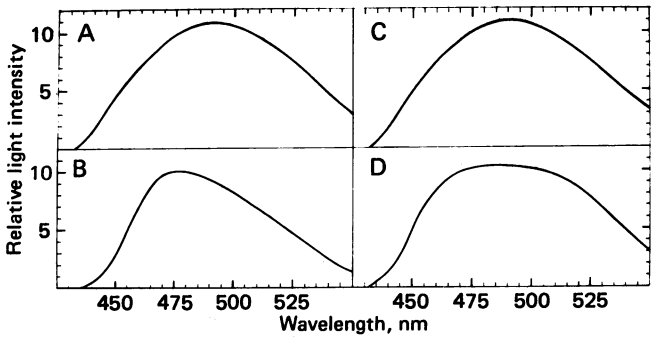

FIG. 4. (*A*) *In vitro* bioluminescence of luciferase from the bacterium type A-13. (*B*) Same as *A*, with addition of blue fluorescence protein. (*C*) *In vitro* bioluminescence with luciferase from the bacterium type *B. harveyi*. (*D*) Same as *C*, with addition of blue fluorescence protein. Reaction conditions are described in the text.

The reaction of Fig. 4 *A* and *B* uses the luciferase from type A-13, the one from which the blue fluorescence protein was isolated. Fig. 4 *C* and *D* shows an attempt at crossreaction between the blue fluorescence protein from A-13 and the luciferase from another species of bioluminescent bacterium, *B. harveyi*. At the concentration added (~70 μM), the spectrum is certainly altered, indicating some crossreaction, but it is not completely shifted over to the A-13 *in vivo* spectrum. Although we can isolate blue fluorescence protein from other species of luminous bacteria, it is not yet available in sufficient quantity for investigation.

The fluorescence properties of the blue fluorescence protein are easily perturbed by a variety of mildly denaturing conditions, such as dilution, temperature, pH, ionic strength, and urea. At high concentration the fluorescence is the same as shown in Fig. 5 for 17 μM, but as the concentration is reduced down to 1 μM, the fluorescence maximum shifts from 474 to 484 nm, with a small reduction in quantum yield. This effect

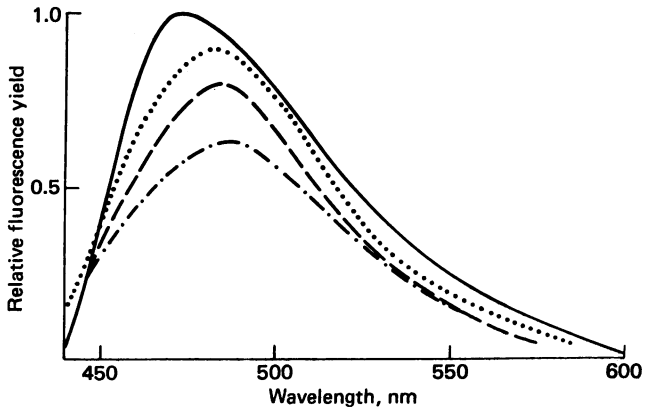

FIG. 5. Fluorescence spectrum of the blue fluorescence protein: (—) 17 μM; (····) 1.75 μM; (- - - -) 0.88 μM; (- · - · -) 0.44 μM. All in 50 mM phosphate, pH 7.0, at 23°; excitation at 420 nm.

is reversible. Below 1 μM the fluorescence shifts only slightly further to the red, where it is now identical to the *in vitro* bioluminescence, but this is accompanied by considerable loss of fluorescence yield, not entirely recoverable on reconcentration. Also, at low concentration (0.5 μM) the fluorescence is completely lost on dialysis, whereas at 10 μM it is quantitatively retained.

The fluorescence quantum yield was measured with fluorescein (0.1 M NaOH, ϕ_F 0.9) and quinine (0.5 M H_2SO_4, ϕ_F 0.55) as standards. At 17 μM, $\phi_F = 0.45$ and the fluorescence lifetime (τ) is 11.1 ns; the extinction coefficient of the chromophore may be calculated by the approximation, $\epsilon = 10^{-4}\ \phi_F/\tau = 4054\ M^{-1}\ cm^{-1}$ (418 nm). The fluorescence polarization is 0.17, in exact prediction of the Weber–Perrin equation (36). At the low concentration end (0.5 μM) the fluorescence lifetime reduces to 8.4 ns and the polarization drops to around 0.10 ($\pm$0.02).

As well as shifting the emission spectrum of the *in vitro* reaction towards the blue, the blue fluorescence protein changes the kinetics and increases the total light. Table 1 shows that at the highest concentration of blue fluorescence protein used the steady-state rate of the light reaction, as measured by the initial light intensity, is increased 6-fold. Without luciferase the blue fluorescence protein has negligible bioluminescence activity. Also, this stimulation is species specific, since the blue fluorescence protein (from type A-13, *P. phosphoreum*) does not stimulate the activity of the luciferase from two other species, *P. fischeri* and *B. harveyi*. Some stimulation of luciferase activity also occurs nonspecifically with protein concentration, such as bovine serum albumin, but this effect is small, and much smaller on a weight basis than for blue fluorescence protein. Higher concentrations of bovine serum albumin inhibit. Heat-denatured blue fluorescence protein produces approximately the same effect as bovine serum albumin.

A reciprocal plot of the data in Table 1 shows that a maximum stimulation of the initial light intensity of about 15 times could be achieved at saturating concentrations of blue fluorescence protein. It has a K_m for stimulation of about 60 μM. The change in spectral distribution on addition of the blue fluorescence protein (Fig. 4 *A* and *B*) affects the calibration factor of the photometer, and this has been taken into account in calculating the data.

The blue fluorescence protein also changes the first-order rate of decay of light intensity, by a factor of two at the highest concentration tested here. Combined with the initial light intensity data, the total light is increased 4-fold at a saturating level of blue fluorescence protein, with a K_m for this interaction of 25 μM.

DISCUSSION

To qualify as the emitter in a bioluminescence system, a chromophore must have a fluorescence spectrum that is the same as the bioluminescence and give evidence of some role in the emission process. Specifically, for bacterial bioluminescence, the shift in the emission spectrum on going from the *in vivo* to the *in vitro* reaction, observed for most types of bacteria (35), must also be explained. The blue fluorescence protein reported here fulfills all these requirements. It has an efficient fluorescence exactly matching the *in vivo* bioluminescence spectral distribution, and the spectrum is readily perturbable by changing conditions, such as its concentration, to exactly match the *in vitro* bioluminescence. It is isolated from extracts of luminous bacteria and clearly participates in the emission process, since on addition to the *in vitro* reaction it shifts the *in vitro* bioluminescence spectrum to that characteristic of its own fluorescence, enhances the rate of photon output and rate of decay of light intensity, and increases the specific light yield of the luciferase.

Chemiluminescence reactions can be divided into two main types, one "direct," in which the light emission comes from a fluorescent product molecule formed directly in its excited state, and the other "indirect" or "sensitized," in which the primary excited species induces the fluorescence of another chromophore (the sensitizer) already present in the mixture. Since exogenous blue fluorescence protein alters the reaction as described above, it is evident that this is a sensitized chemiluminescence process. Murphy *et al.* (37) suggested that bacterial bioluminescence was a sensitized chemiluminescence when they found that, on reaction of bacterial luciferase with $FMNH_2$ and O_2, an intermediate was formed which was separated from all flavin yet retained full bioluminescence activity on reaction with aldehyde. We have made similar observations (38). It is also interesting that Cormier and Kuwabara (11) were able to find a significant bioluminescence activity of reduced neutral red with their crystalline luciferase preparations in the absence of any detectable flavin. Sensitization specifically by energy transfer has recently been proposed by Ruby and Nealson (18) to account for a 545 nm *in vivo* bioluminescence maximum in a bacterium they isolate, whereas the *in vitro* maximum was again at 495 nm.

Two other bioluminescence systems that utilize aldehyde as their substrate may also be sensitized. These are the fresh water limpet, *Latia neritoides* (39), and the earthworm, *Diplocardia longa* (40). Although in these systems no direct evidence for a sensitizer has been obtained by isolation, oxidation of the aldehyde alone would not be expected to yield a fluorescent molecule. A better characterized sensitized bioluminescent system is that of the coelenterates, where the emission is sensitized by the addition of a "green fluorescent protein" (41). There are clear differences in the properties of these two systems, however, the first being that the coelenterate reaction is efficiently sensitized by green fluorescent protein at a concentration about one-tenth that (42) of the blue fluorescence protein in the bacterial reaction (Table 1). Although a 4-fold increase in light yield occurs in both systems, the overlap of the absorption of blue fluorescence protein with the emission spectrum from the bacterial *in vitro* reaction is two orders of magnitude less than that of the green f uorescent protein with the coelenterate *in vitro* bioluminescence, and moreover, the shift in the bacterial case is to a higher energy.

It is also significant that the fluorescence spectrum of the blue fluorescence protein can itself be suitably perturbed to match either the *in vivo* or the *in vitro* bioluminescence. The effect of concentration on its fluorescence properties can be accounted for by the equilibrium:

$$BFP_{474} \rightleftarrows BFP_{490} \rightleftarrows BF + P$$

with a dissociation constant 10^{-7} M for the chromophore "BF" from the protein. This chromophore is less stable and nonfluorescent in free solution and is dialyzable below 0.5 μM, but not at higher concentration. Dilution of the blue fluorescence protein BFP_{474} therefore pulls the equilibrium to the redder form BFP_{490}, in which its lowered fluorescence polarization suggests that the chromophore remains protein-bound but is less constrained to rotation than in BFP_{474}. This result is also consistent with the chromophore being more exposed to the water, a more polar environment inducing the red shift. The other perturbing agents (temperature, pH, etc.) produce a similar effect and can be explained by the same model.

The molecular structure of the chromophore in the blue

fluorescence protein is not known. It does not appear to be derived from flavin (43). There is a similarity to the absorption and fluorescence characteristics of a NAD^+-aldehyde adduct described by Cormier *et al.* (13).

Although our results bear primarily on the nature of the emitter in the *in vivo* reaction, the demonstrated ability of exogenous blue fluorescence protein to sensitize the *in vitro* reaction leads to a reappraisal of the identities both of the primary excited state and of the emitter in the reaction of luciferase alone.

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1. Strehler, B. L. (1953) *J. Am. Chem. Soc.* **75,** 1264.
2. McElroy, W. D. (1961) in the *The Bacteria 2 Metabolism,* eds. Gunsalus, I. C. & Stanier, R. Y. (Academic Press, New York), pp. 479–508.
3. Lee, J. & Murphy, C. L. (1973) in *Chemiluminescence and Bioluminescence,* eds. Cormier, M. J., Hercules, D. M. & Lee, J. (Plenum Press, New York), pp. 381–386.
4. Cormier, M. J. & Eckroade, C. B. (1962) *Biochim. Biophys. Acta* **64,** 340–344.
5. Cormier, M. J. & Totter, J. (1964) *Annu. Rev. Biochem.* **33,** 431–458.
6. Terpstra, W. (1962) *Biochim. Biophys. Acta* **60,** 580–590.
7. Terpstra, W. (1963) *Biochim. Biophys. Acta* **75,** 355–364.
8. Kuwabara, S., Cormier, M. J., Dure, L. S., Kreiss, P. & Pfuderer, P. (1965) *Proc. Natl. Acad. Sci. USA* **53,** 822–828.
9. Hastings, J. W., Riley, W. H. & Massa, J. (1965) *J. Biol. Chem.* **240,** 1473–1481.
10. Lee, J., Murphy, C. L., Faini, G. J. & Baucom, T. L. (1974) in *Liquid Scintillation Counting, Recent Developments,* eds. Stanley, P. E. & Scoggins, B. A. (Academic Press, New York), pp. 403–420.
11. Cormier, M. J. & Kuwabara, S. (1965) *Photochem. Photobiol.* **4,** 1217–1225.
12. Eley, M., Lee, J., Lhoste, J.-M., Lee, C. Y., Cormier, M. J. & Hemmerich, P. (1970) *Biochemistry* **9,** 2902–2908.
13. Cormier, M. J., Eley, M., Abe, S. & Nakano, Y. (1969) *Photochem. Photobiol.* **9,** 351–358.
14. Mitchell, G. & Hastings, J. W. (1969) *J. Biol. Chem.* **244,** 2572–2576.
15. McCapra, F. & Hysert, D. W. (1973) *Biochem. Biophys. Res. Commun.* **52,** 298–304.
16. Balny, C. & Hastings, J. W. (1975) *Biochemistry* **14,** 4719–4723.
17. Tu, S. & Hastings, J. W. (1975) *Biochemistry* **14,** 1975–1980.
18. Ruby, E. G. & Nealson, K. H. (1977) *Science* **196,** 432–434.
19. Reichelt, J. L. & Baumann, P. (1973) *Arch. Mikrobiol.* **94,** 283–330.
20. Lee, J. (1972) *Biochemistry* **11,** 3350–3359.
21. Lee, J. & Murphy, C. L. (1975) *Biochemistry* **14,** 2259–2268.
22. Lee, J., Wesley, A. F., Ferguson, J. F. & Seliger, H. H. (1966) in *Bioluminescence in Progress,* eds. Johnson, F. H. & Haneda, Y. (Princeton University Press, Princeton, NJ), pp. 35–43.
23. Lee, J. & Seliger, H. H. (1965) *Photochem. Photobiol.* **4,** 1015–1048.
24. Fontijn, A. & Lee, J. (1972) *J. Opt. Soc. Am.* **62,** 1095–1098.
25. Heller, C. A., Carlisle, D. T. & Meary, R. A. (1971), *J. Lumin.* **4,** 81–88.
26. Michael, P. R. & Faulkner, L. R. (1976) *Anal. Chem.* **48,** 1188–1192.
27. Wampler, J. E. (1978) in *Bioluminescence in Action,* ed. Herring, P. J. (Academic Press, London), in press.
28. Ware, W. R. (1971) in *Creation and Detection of Excited States,* ed. Lamola, A. A. (Marcell Dekker, New York), Vol. 1A, pp. 213–302.
29. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412.
30. Hastings, J. W., Weber, K., Friedland, J., Eberhard, A., Mitchell, G. W. & Gunsalus, A. (1969) *Biochemistry* **8,** 4681–4689.
31. Brewer, J. & Ashworth, R. B. (1969) *J. Chem. Ed.* **46,** 41–45.
32. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
33. Spruit-van der Burg, A. (1950) *Biochim. Biophys. Acta* **5,** 175–178.
34. Lee, J. (1977) in *The Science of Photobiology,* ed. Smith, K. C. (Plenum Press, New York), pp. 371–395.
35. Seliger, H. H. & Morton, R. A. (1968) in *Photophysiology,* ed. Giese, A. C. (Academic Press, New York), Vol. IV, pp. 253–314.
36. Weber, G. (1953) *Adv. Protein Chem.* **8,** 415–459.
37. Murphy, C. L., Faini, G. & Lee, J. (1974) *Biochem. Biophys. Res. Commun.* **58,** 119–125.
38. Gast, R. & Lee, J. (1975) *American Society for Photobiology Abstracts* (American Society for Photobiology), p. 43.
39. Shimomura, O., Johnson, F. H. & Kohama, Y. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2086–2089.
40. Ohtsuka, H., Rudie, N. G. & Wampler, J. E. (1976) *Biochemistry* **15,** 1001–1006.
41. Cormier, M. J., Lee, J. & Wampler, J. E. (1975) *Annu. Rev. Biochem.* **44,** 255–272.
42. Ward, W. W. & Cormier, M. J. (1976) *J. Phys. Chem.* **80,** 2289–2291.
43. Gast, R. & Lee, J. (1976) *Int. Congress on Photobiol., Rome, Italy, Abstracts,* p. 324.