Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 14-21

SEPARATION OF A BLUE FLUORESCENCE PROTEIN FROM BACTERIAL LUCIFERASE

Robert Gast, Ian R. Neering and John Lee

Department of Biochemistry
University of Georgia
Athens, Georgia 30602

Received November 7,1977

SUMMARY: Luciferase preparations from two species of marine bioluminescent bacteria, Photobacterium phosphoreum and Photobacterium fischeri, are shown to contain a low molecular weight protein, containing a blue fluorescence chromophore having an emission maximum in the 470 nm region. A procedure for separating the luciferase and purifying this protein is described. On disc gel electrophoresis the bulk of the protein is observed to migrate along with the blue fluorescence.

The blue emission of bioluminescent bacteria is a broad spectrum with a maximum intensity around 490 nm for most types of bacteria (1). The bioluminescence reaction in vitro requires an enzyme, bacterial luciferase, with substrates reduced FMN, oxygen and a long chain aliphatic aldehyde, such as dodecanal. There has been a continuing search over the years for some substance in the reaction having fluorescent properties which would account for the bioluminescence emission, either a chromophore attached to the luciferase (2-6) or one formed by the reaction (4,7,8).

Recently the isolation of a blue fluorescence protein (BFP) from preparations of the luciferase from Photobacterium phosphoreum, was announced (9). The fully corrected fluorescence emission spectrum of the BFP was an exact match for the in vivo bioluminescence from P. phosphoreum, and in the presence of various mildly denaturing agents the spectrum could be shifted 15 nm to longer wavelengths to exactly match the in vitro bioluminescence spectrum using P. phosphoreum luciferase. The addition of BFP to the in vitro reaction also affected the light emission kinetics, increased the light yield and induced a

0006-291X/78/0801-0014$01.00/0

shift to shorter wavelengths in the bioluminescence emission. These authors suggested that BFP itself was the in vivo bioluminescence emitter (9).

In this present work we describe the separation and partial purification of BFP from the luciferase preparations of two species of bacteria, P. phosphoreum and P. fischeri.

EXPERIMENTAL

The bacteria were P. phosphoreum strain "A13" from J. Fitzgerald (Monash University) and P. fischeri, strain 399 in the numbering system of Reichelt and Baumann (10). Bacteria were maintained on a solid agar medium and grown in 400 l liquid batches for luciferase preparation (11). Luciferase was assayed by rapid injection of $FMNH_2$ (0.5 ml, 80 μM) to the sample in buffer (1 ml) containing bovine serum albumin (1 mg) and dodecanal (10 μl of a saturated solution in methanol), all at 23°C. The maximum light intensity denotes the luciferase activity, and was measured by a photometer calibrated for absolute photon sensitivity by reference to the luminol chemiluminescence quantum yield standard (12). The BFP was assayed by measuring the fluorescence intensity of a solution in buffer, using an Aminco-Bowman spectrofluorimeter, with the emission wavelength set at 470 nm and the excitation set at the maximum 420 nm for P. phosphoreum and 410 nm for P. fischeri. The absorbance at the excitation wavelength was adjusted to below 0.1 to avoid attenuation of the fluorexcence signal due to self-absorption. Linearity of fluorescence intensity and concentration of BFP was established and a fluorescein standard solution was used to maintain a constant day-to-day instrumental sensitivity. The concentration of BFP is reported here in arbitrary fluorescence units. Absorption measurements were made on a Cary 14 spectrophotometer. All preparative procedures (see Table) were made at below 5°C, in a buffer of 0.05 M phosphate, 0.3 mM EDTA, pH 7.0. All chemicals were of the best commercial grades. The alkaline disc gel electrophoresis (13) was carried out at 5°C in 7.5% acrylamide containing 1 mM dithiothreitol.

RESULTS AND DISCUSSION

Cells were cultured at room temperature for convenience and harvested in the vicinity of a cell density yielding maximum light per cell. For P. phosphoreum a substantial increase in light per cell and yield of BFP can be obtained by growth at 12°C.

The processing of the cell lysate is given in the Table. About 500 g of wet cell cake was suspended in buffer (1 l) containing dithiothreitol (1 mM) and phenymethylsulfonylfluoride (1 μM) to retard proteolysis, and disrupted by two passages through a French press. Cell walls were removed by centrifugation (60 min, 25000 g) then partial fractionation made by addition over 30 min of ammonium sulfate to 30% saturation, then centrifuging (30 min, 25000 g)

Table. Purification of the Blue Fluorescence Protein (BFP).

	Total Bioluminescence Activity[a] 10^{12} photons-s^{-1}-ml^{-1}	Total Fluorescence[a] 420 ⟶ 470 nm (arbitrary units)	Total Absorbance at 280 nm[a]
A. *P. phosphoreum*			
1. Cell lysate	4100	3700[b]	47300
2. Ammonium sulfate fractionation			
30% supernatant	10300	600	59600
80% pellet	7000	540	46600
3. Sephadex G75			
luciferase fraction	7200	124	23600
free BFP fraction[c]	(130)	(210)	(3300)
4. DEAE-cellulose, 0.15 M eluate	7200	30	1700
5. DEAE-Sephadex A50 (0.22 - 0.28 M)	4400	23	780
6. Sephadex G75 superfine			
luciferase fraction	3500	2	133
free BFP fraction	0.1	6	54
B. *P. fischeri*			
1. Cell lysate	43400	490	65000
2. Ammonium sulfate fractionation			
30% supernatant	39200	320	38700
80% pellet	31600	250	35200
3. Sephadex G75			
luciferase fraction	30000	44	12500
4. DEAE-cellulose, 0.25 M eluate	24200	26	2000
5. DEAE-Sephadex A50			
0.25 M fraction	5	16.5	354
0.35-0.5 M fraction	26000	0.9	414
6. Sephadex G75 superfine			
free BFP fraction	6.5	22	223

a. Totals are the luciferase activity/ml x total volume (similarly for fluorescence and A(280). b. This includes a major contribution due to light scattering. c. Not further processed.

The supernatant contained all the luciferase activity (some material in the lysate appears to inhibit the assay) and the pellet was discarded. The supernatant was then made 80% saturation in ammonium sulfate and centrifuged (30 min, 25000 g) after two or more hours.

The amount of true fluorescence in the lysate cannot be estimated due to the major contribution of light scattering. The BFP concentration was therefore estimated here by chromatographing a small sample of the 30% supernatant on a column of Sephadex G75 superfine grade, thereby separating out the BFP from other fluorescent components. About 20% of the fluorescence (Table , line 2), that is about 120 fluorescence units in the 30% supernatant, can be attributed to BFP. For P. fischeri the percentage is about the same.

The third step is desalting by gel filtration in preparation for ion-exchange chromatography. The 80% pellet was redissolved in a minimum amount of buffer (200 ml) and applied to a column of Sephadex G75 (10 x 60 cm). The elution pattern (Fig. 1) shows the luciferase activity at a volume 600 ml almost at the column front, as expected from its molecular weight of 82,000 (5). The fluorescence spectra of fractions in the range 300-1600 ml have maxima around 470 nm corresponding to BFP (9). The material at 2000 ml has a flavin-like fluorescence with a maximum around 520 nm (uncorrected). Some BFP is seen to elute with the luciferase fraction (400-900 ml), possibly an association between these two proteins. However most of the BFP separates and is retarded to around 1400 ml, as expected from its molecular weight of approximately 20,000 (9). An efficient method of purification of this free BFP (1100-1600 ml, Fig. 1) will be described elsewhere as this present work is concerned only with the luciferase-associated BFP. As far as we can tell, the properties of BFP prepared by either route are the same. With P. fischeri (Table B), 95% of the BFP remains luciferase-associated at this stage.

The two anion-exchange steps (4 and 5) were carried out at pH 7.6 for P. phosphoreum and 7.0 for P. fischeri. For P. phosphoreum the luciferase fraction from step 3 (e.g. Fig. 1, 250-1050 ml) was loaded to DEAE-cellulose

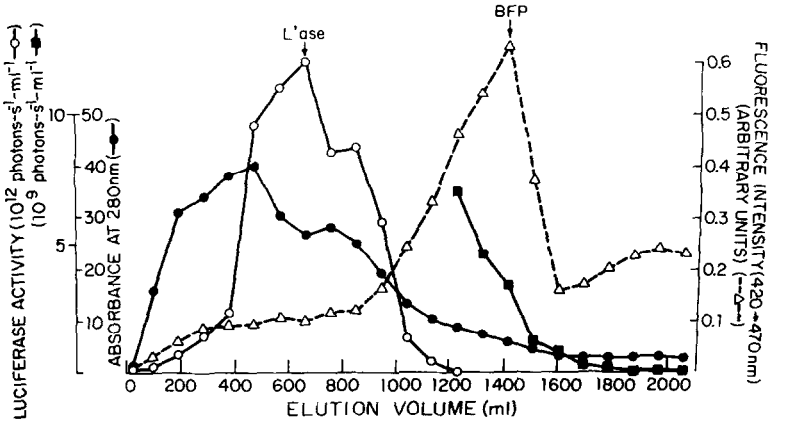

Figure 1. Elution of crude P. phosphoreum luciferase (L'ase) on Sephadex G75 (arbitrary zero), 2 ml/min. Column front is around the 500 ml mark. Some Blue Fluorescence Protein (BFP) co-elutes with the luciferase but most is retarded to around 1400 ml (mol. wt. ~ 20,000).

(DE-32, 6 x 20 cm). The column was washed with buffer (500 ml) and the luciferase/BFP batch-eluted with higher phosphate (0.15 M, 500 ml). This eluate was diluted 1.5-2 fold with distilled water to reduce the salt concentration and loaded to DEAE-Sephadex (A-50, 2 x 20 cm). This column was washed (0.1 M phosphate, 500 ml) and eluted with a linear phosphate gradient (0.15 - 0.3 M, 500 x 500 ml). There was a slight separation between BFP (maximum fluorescence at salt concentration 0.24 M) and luciferase (0.27 M), but the separation was inefficient and all the fractions 0.22 - 0.28 M were therefore combined for filtration on Sephadex G75 superfine grade (4.5 x 100 cm). Elution was made with buffer containing 2-mercaptoethanol (2 mM).

Figure 2 shows that this slow molecular sieving completely separated the P. phosphoreum luciferase which elutes in the front around 250 ml, from the BFP (maximum at 520 ml). The absorbance at 450 nm is maximum at an elution volume of 310 ml and corresponds to a third, highly colored component which, from its characteristic absorption spectrum appears to be a flavoprotein.

The ion exchange steps of P. fischeri required a higher salt concentration, Table B steps 4 and 5. Step 5 in fact provided good separation of BFP in

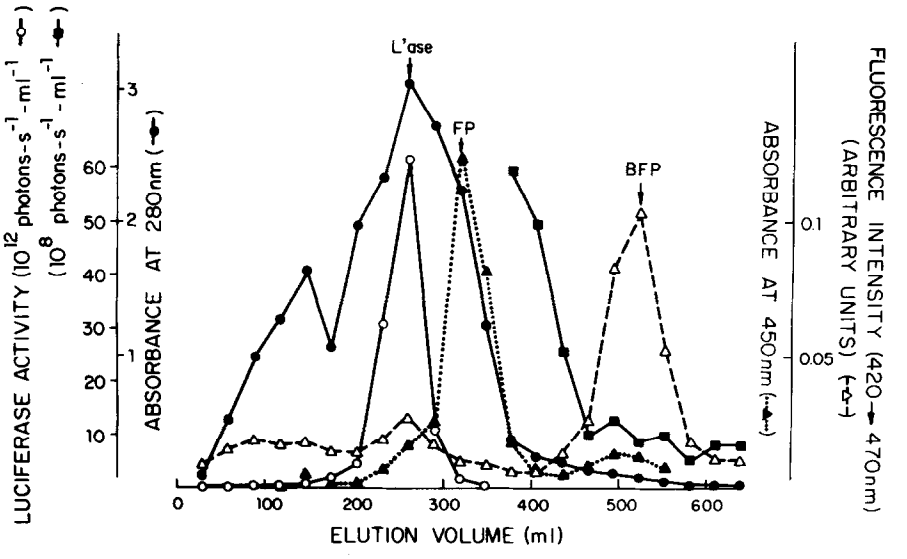

Figure 2. Separation of P. phosphoreum luciferase (260 ml) from blue fluorescence protein (BFP, 520 ml) on Sephadex G75 superfine (10 ml/hr.). A non-fluorescent flavoprotein (FP) appears at 310 ml.

the 0.25 M fraction, from the luciferase. This free BFP was then applied to the slow sieving column, step 6, and eluted in a volume about the same as for P. phosphoreum BFP, suggesting similar molecular weights.

If the figure of 20% of the fluorescence of the 30% supernatant, step 2 in the Table, is used to estimate the BFP in the lysate, then the purification of BFP through this last stage is 50-100 fold, with a yield of 6%, (P. phosphoreum) and 30% (P. fischeri). Two contaminating fluorescent components appear in the BFP preparation, arising from proteins of apparently similar molecular weights. One has a flavin-like fluorescence, the spectrum showing a 520 nm (uncorrected) shoulder when excited at 470 nm. This component is progressively removed by repeated chromatography on Sephadex G75 superfine.

The second fluorescent impurity has a maximum at 420 nm (uncorrected) when excited at 370 nm. It may be a denaturation product of the BFP since its fluorescence intensity increases at the expense of the 420 ⟶ 470 nm fluorescence if the BFP solution is allowed to stand over several days (5°C). If the BFP solution is applied to a DEAE-cellulose column (DE-32) pH 6.5, phosphate

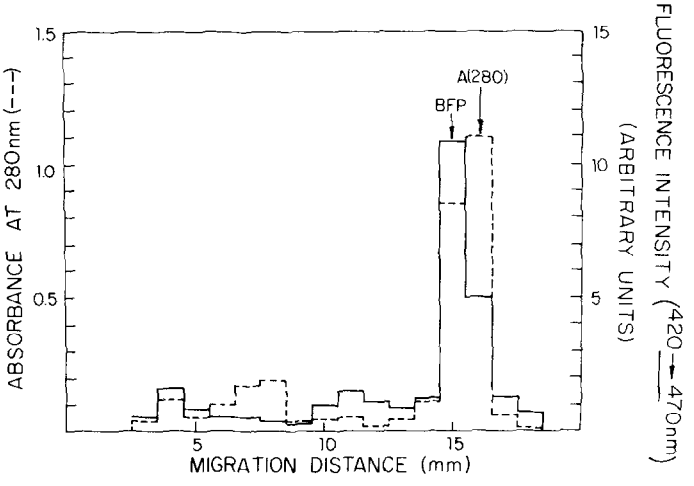

Figure 3. Alkaline disc gel electrophoresis of P. phosphoreum BFP (fraction at 520 ml, Fig. 2).

0.05 M, the BFP can be eluted with 0.1 M phosphate (pH 6.5) and the 420 nm fluorescent material remains bound.

Disc Gel Electrophoresis. Protein from the peak BFP fractions off the Sephadex column Fig. 2 (step 6) was subject to a disc gel analysis, and Fig. 3 shows that the bulk of the protein has the same electrophoretic properties as the 420 ⟶ 470 nm fluorescent material. The gel was sliced into 1 mm sections, extracted into buffer (20 hrs) and then assayed for fluorescence and absorbance against a reference blank gel extract. The maxima of absorbance and fluorescence do not differ significantly and have an average R_F of 0.8. About 70% of the total 280 nm absorbance is included under the fluorescent band. There are no other visible fluorescent protein bands. On further chromatography on the Sephadex G75 superfine, the protein purity is increased to about 90%.

The fluorescence and absorption characteristics of the BFP from P. fischeri suggest a similar degree of purity.

Although the BFP's from these two species of bacteria appear to have similar molecular weights, they differ in that P. fischeri BFP is more tightly

associated with its luciferase and it has a fluorescence excitation maximum shifted about 10 nm to shorter wavelength. Gast and Lee (9) have proposed that BFP has a function in the bacterial bioluminescence reaction. The present evidence for the presence of BFP's having similar properties in two of the four common species of marine bioluminescent bacteria, would appear to add further support to this hypothesis.

ACKNOWLEDGEMENTS. We thank James Diguiseppi for his technical assistance and Dr. William Ragland for use of his gel fluorescence scanner. This work was supported by grants from NSF, BMS 74-19890 and NIH, GM19163.

REFERENCES

1. Seliger, H. H., and Morton, R. A. (1968) in "Photobiology" (Giese, A. C., ed.) Vol. IV, pp. 253-314, Academic Press, N. Y.
2. Terpstra, W. (1962) Biochim. Biophys. Acta 60, 580-590.
3. Mitchell, G. W., and Hastings, J. W. (1970) Biochemistry 9, 2699-2707.
4. Eley, M., Lee, J., Lhoste, J.-M., Lee, C. Y., Cormier, M. J., and Hemmerich, P. (1970) Biochemistry 9, 2902-2908.
5. Nakamura, T. and Matsuda, K. (1971) J. Biochem. 70, 35-44.
6. Tu, S., and Hastings, J. W. (1975) Biochemistry 14, 1975-1980.
7. Terpstra, W. (1963) Biochim. Biophys. Acta 75, 355-364.
8. Balny, C., and Hastings, J. W. (1965) Biochemistry 14, 4719-4723.
9. Gast, R., and Lee, J. (1976) Int. Congress on Photobiol. Abstracts p. 324.
10. Reichelt, J. L. and Baumann, P. (1973) Arch. Mikrobiol. 94, 283-330.
11. Lee, J. (1972) Biochemistry 11, 3350-3359.
12. Lee, J., Wesley, A. F., Ferguson, J. F. and Seliger, H. H. (1966) in "Bioluminescence in Progress" (Johnson, F. H. and Haneda, Y., eds.) pp. 35-43, Princeton University Press, N. J.
13. Brewer, J. M. and Ashworth, R. B. (1969) J. Chem. Educ. 46, 41-45.