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HIGH MOLECULAR WEIGHT BLUE FLUORESCENCE PROTEIN FROM THE BIOLUMINESCENT BACTERIUM *Photobacterium fischeri*

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SUMMARY: A blue fluorescence protein has been purified from extracts of the bioluminescent bacterium *Photobacterium fischeri* and found to have a native molecular weight of 70,000, and to be a dimer of two identical subunits. SDS gel electrophoresis distinguishes the monomer from the two non-identical subunits of luciferase. The molecular weight for this blue fluorescence protein contrasts with the much lower value (22,000) reported for the same type of protein isolated from *Photobacterium phosphoreum*.

Significant quantities of a blue fluorescence protein partially associated with luciferase, have been found to be produced by the bioluminescent bacterium *Photobacterium phosphoreum* (1-3). This novel protein has been partially characterized and found to have a molecular weight of 22,000 (2,4). Following purification to homogeneity using affinity chromatography on Blue Sepharose (4) the fluorophore prosthetic group was separated and found to have spectroscopic properties identical to 6,7-dimethyl-8-ribityl lumazine (5). Indeed the absorption and fluorescence spectral maxima of the blue fluorescence protein itself are very similar to the spectral maxima of this lumazine bound to riboflavin synthetase, for which it is the substrate in the biosynthesis of riboflavin (6).

The blue fluorescence protein has a role in the bioluminescence of *P. phosphoreum*. Its fluorescence spectral distribution is identical to the *in vivo* bioluminescence and under certain solution conditions it can shift its fluorescence 15 nm to the red, where it is a match for the *in vitro* bioluminescence spectrum (2). But when added to this luciferase reaction it blue shifts the spectrum back to

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that matching the *in vivo*, as well as having stimulatory effects on the bioluminescence kinetics and light yield (1).

Gast and Lee (1) have suggested that the blue fluorescence protein acts in this bacterium as a sensitizer of the bioluminescence and that it is the *in vivo* emitter. It is not known whether other species of bioluminescent bacteria function in this way but it would be supportive of the idea if a blue fluorescence protein could also be isolated from them. In fact such a protein has been found in *Photobacterium fischeri* (3).

Since the preliminary report it has been observed that high concentrations of 2-mercaptoethanol are necessary to prevent denaturation of the *P. phosphoreum* blue fluorescence protein in the fraction that is not associated with luciferase (4), and of the blue fluorescence protein from *P. fischeri*. As a consequence the molecular weight of the *P. fischeri* type in the native state is shown here to be 70,000, in contrast to the 22,000 value for that from *P. phosphoreum*.

MATERIALS AND METHODS

The purification of blue fluorescence protein from *P. fischeri* was by the method already described (3) except the standard buffer 50 m*M* phosphate pH 7.0 contained 10-15 m*M* 2-mercaptoethanol and changes were made in the chromatographic materials to provide faster flow rates and more efficient separation. In the first ion exchange step, DEAE-Sephacel (Pharmacia) was used in place of DEAE-cellulose and the molecular sieving was done on Sephacryl S200 Superfine (Pharmacia) instead of Sephadex G75 Superfine. All concentrations were done by ultrafiltration using an Amicon (Lexington, Mass.) ultrafiltration device, PM-30 membrane, 30,000 molecular weight cut-off.

Following the Sephacryl column was added an affinity chromatography step (4,5). Cibacron Blue-Sepharose 4B (Blue Sepharose) was prepared by the method of Travis *et al.* (7): 4-5 g of Cibacron Blue F-3-GA (Polysciences, Warrington, Pa.) combined with 500 ml Sepharose CL-4B (Pharmacia). The fractions off Sephacryl S200 were concentrated to 5 ml and loaded to the Blue Sepharose column (1.5 x 20 cm)

prewashed with standard buffer (200 ml), then washed with standard buffer (200 ml) and eluted by 0.1 *M* phosphate (200 ml) followed by 0.15 *M* phosphate (200 ml), each containing 2-mercaptoethanol (10 m*M*).

Sedimentation equilibrium studies were made on a Beckman Model E ultracentrifuge equipped with absorption optics. The rotor was AN-HT1 and the windows were double sector sapphire. Samples were spun for 24 hours at 5°C or room temperature, at 8,000 or 14,000 rpm.

Polyacrylamide gel electrophoresis was made using sodium dodecyl sulfate (SDS) "specially pure" (BDH Chemicals, Poole, England). For the molecular weight determination, electrophoresis was done in the discontinuous buffer system of Neville (8). The upper buffer was as described by Neville (8) while the lower gel buffer pH 9.18 consisted of 0.031 *M* HCl - 0.42 *M* Tris. The lower gel was 10% acrylamide, 0.3% bisacrylamide. Polymerization was done by 0.2% N,N,N,N-tetramethylethylenediamine and ammonium persulfate (0.9 mg/ml). The proteins used as standards were α-chymotrypsin, carbonic anhydrase, enolase, ovalbumin, bovine serum albumin and phosphorylase a. To compare luciferase and the blue fluorescence protein, SDS gel electrophoresis was done by the method of Weber and Osborn (9) using 10% acrylamide gels. The stain used for both systems was 0.1% Coomassie G250 (Bio-Rad) in 7% acetic acid.

All reagents were of the best commercial grade.

RESULTS

The first indication that the molecular weight of this blue fluorescence protein from *P. fischeri* was higher than the preliminary estimate, came at the slow molecular sieving step. The 410→470 nm fluorescing material (Fig. 1) co-eluted with the small amount of luciferase activity (mol. wt. 79,000) (10) carried along from the previous ion-exchange step.

The protein was not homogeneous at this stage and also showed some flavin-like fluorescence when excited at 470 nm. The 345-400 ml volume from Fig. 1 was concentrated and applied to the Blue Sepharose column, and the result was a homo-

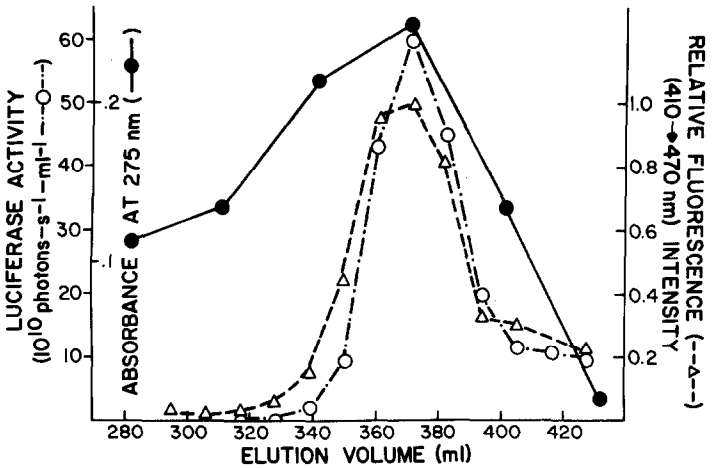

Figure 1. Elution of *P. fischeri* luciferase (-o-) and blue fluorescence protein (-Δ-) on Sephacryl S200 superfine (25 ml/hr). There is co-elution of the two proteins.

geneous protein of lowest ratio of absorbance at 277 nm to 408 nm, comparable to the absorption spectrum of the blue fluorescence protein from *P. phosphoreum* (1,5)

The Sephacryl column was calibrated with the molecular weight markers: luciferase (*P. fischeri*), catalase, enolase, bovine serum albumin and horseradish peroxidase. The purified blue fluorescence protein eluted again at the same volume as luciferase, *i.e.* it has the same Stokes' radius and therefore an approximate molecular weight of 79,000 (10).

The protein was concentrated, rediluted without 2-mercaptoethanol and reconcentrated, using an Amicon Minicon (B-15), for study by equilibrium centrifugation. Figure 2 is one set of results plotted in the standard manner (Y is proportional to absorbance) for the two absorption maxima. Both plots are linear consistent with protein homogeneity and the slopes are the same, meaning that the chromophore absorbing at 408 nm is attached to a species with the same molecular weight as the bulk of the protein in the sample. Assuming a partial volume of 0.74 cc-g^{-1}, the average molecular weights and coefficient of variation for three experiments at different concentrations measured at 277 nm is 64,000 $\pm$ 9,000 and for seven experiments at 408 nm, 61,000 $\pm$ 5,000.

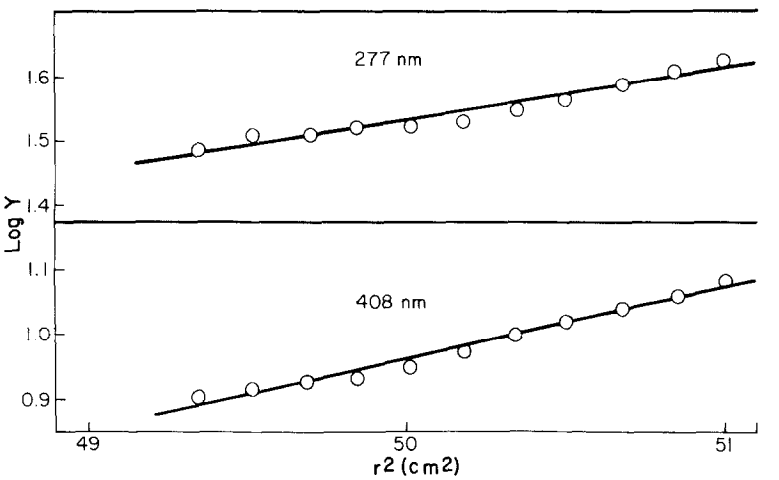

Figure 2. Molecular weight determination by sedimentation equilibrium - plots of log Y versus r^2 at two different wavelengths. The similar slopes suggest that the chromophore absorbing at 408 nm is bound to the protein absorbing at 277 nm. (Y is proportional to concentration, r is the radial distance).

Figure 3 is the SDS gel electrophoresis. It shows that the *P. fischeri* blue fluorescence protein sample in the left gel consists of a single band and minor contaminants are detectable only when the total protein applied exceeds 20 µg. When applied with an equal amount of *P. fischeri* luciferase in the middle gel, three bands are seen. Assuming 41,000 and 38,000 for the two subunits of luciferase (10) this gives an estimate of about 35,000 for the monomer molecular weight of the blue fluorescence protein band. In the right gel the Neville technique (8) is used and two markers, phosphorylase a (94,000) and enolase (44,000) are shown along with the blue fluorescence protein (lowest band). A series of runs were made with these and the other markers (see Methods) and the results plotted by the Neville method (8), yielding a molecular weight of 38,000 for this blue fluorescence protein.

DISCUSSION

Since the molecular weight estimate under denaturing conditions is approximately one-half that for the native protein, it is apparent that the blue fluores-

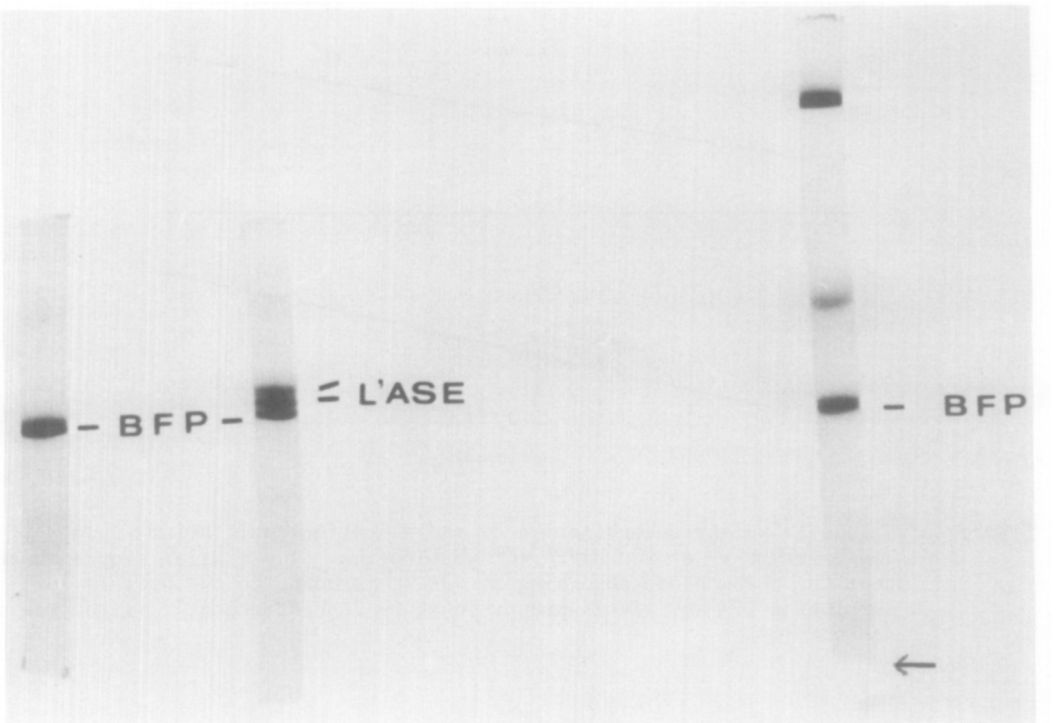

Figure 3. SDS-polyacrylamide gel electrophoresis. *Left:* Blue fluorescence protein (10 µg) showing homogeneity. *Middle:* Luciferase (5 µg) from *P. fischeri* - molecular weights 41,000 and 38,000 - with blue fluorescence protein (5 µg) showing that they are nonidentical. *Right:* Molecular weight determination (Neville, SDS disc method). Markers: phosphorylase a (94,000), enolase (44,000) and blue fluorescence protein (lowest band). The arrow indicates the dye front.

cence protein from *P. fischeri* is a dimer, and the sub-units have the same molecular weight. Doubling these estimates for the subunits and averaging in with the results from gel filtration and equilibrium centrifugation monitored at 277 nm and 408 nm, gives a molecular weight for the native protein of 70,000 (± 4,000).

It is obviously a very different protein from that isolated from *P. phosphoreum* (mol. wt. 22,000) although the coincidence of the absorption spectra (not shown) along with other evidence to be published, suggests the same chemical structure for the prosthetic group.

The similarity of its Stokes' radius to that of luciferase explains why contaminating activity persists in the preparation (<1%). The apoprotein is not luciferase however, as evident from the SDS gel Fig. 3 (middle).

It has recently been suggested by Hastings and Nealson (11) that the blue fluorescence protein from *P. phosphoreum* was identical to photoexcitable luciferase which is a luciferase containing a bound substituted flavin (11). The molecular weight of 22,000 for that blue fluorescence protein (2) to be compared with *P. phosphoreum* luciferase, 82,000 (12) would make that suggestion unlikely. The possibility might be considered in the case of *P. fischeri* described here, but again the clear difference in sub-units would appear to rule it out.

Another chromophoric substance called "p-flavin" has been isolated from *P. phosphoreum* (13). From its characteristic absorption spectrum, it has been reported to be a flavin attached to luciferase, both properties again distinguishing it from the low molecular weight blue fluorescence (lumazine) protein of that same species.

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