Intermolecular Energy Transfer in the Bioluminescent System of *Aequorea*†

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ABSTRACT: The jellyfish *Aequorea* emits green light whereas the photoprotein aequorin extracted from the same organism emits blue light when Ca^{2+} is added. Because the photogenic cells contain a green fluorescent protein (GFP) in addition to aequorin, an energy transfer from the light emitter of aequorin to GFP has been postulated. In the present study, GFP has been purified, crystallized, and partially characterized and an energy transfer *in vitro* from aequorin to this protein has been demonstrated. GFP was found to consist of several kinds of isomeric proteins, of which two kinds predominated. After separation, both kinds evinced the same absorption maxima at 280, 400, and 480 nm, fluorescence emission maximum at 508–509 nm, and fluorescence quantum yield of 0.72 when excited at 470 nm. Addition of Ca^{2+} to aequorin solutions containing a relatively low concentration of GFP resulted in luminescence close to that from aequorin alone (λ_{max} 472 nm). When GFP and aequorin were initially coadsorbed on DEAE-cellulose or DEAE-Sephadex, however, luminescence on adding Ca^{2+} was green (λ_{max} 509 nm) closely corresponding to the *in vivo* luminescence. The protein–protein energy transfer is considered to involve a Förster-type mechanism *in vivo* as well as *in vitro*.

The bioluminescent jellyfish *Aequorea* emits "green light" *in vivo*, whereas the pure photoprotein aequorin extracted from the same organism emits "blue" light on addition of Ca^{2+}. This difference, along with some of the properties of the purified aequorin and also the occurrence of a green fluorescent protein in the luminescent extract, was first reported over 10 years ago (Shimomura *et al.*, 1962).

The blue light (λ_{max} 470 nm) of aequorin (mol wt *ca.* 30,000; Shimomura and Johnson, 1969; Kohama *et al.*, 1971) results from an intramolecular reaction when Ca^{2+} is added, yielding a reaction product "BFP" ("blue fluorescent protein") which shows a fluorescence spectrum corresponding exactly to the bioluminescence spectrum (Shimomura and Johnson, 1970a). The chemical structure of the light emitting chromophore has been recently determined to be 2-(*p*-hydroxyphenylacetyl)-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine (Shimomura and Johnson, 1972, 1973; Kishi *et al.*, 1972).

Although the presence of a green fluorescent substance in the photogenic tissue has been known for a long time (Davenport and Nicol, 1955), it was first recognized to be protein in nature in 1962 (Shimomura *et al.*, 1962; Johnson *et al.*, 1962). Virtually no data concerning this substance have been reported, except for fluorescence spectra as measured on partially purified materials. These measurements indicated an excitation maximum at 460 nm and an emission maximum at 508–515 nm (Johnson *et al.*, 1962; Cormier *et al.*, 1973). Such spectral properties, together with the *in vivo* bioluminescence spectrum of *Aequorea* (green, λ_{max} 508–515), naturally led to the suggestion by several authors that the *in vivo* luminescence involves an energy transfer from the light emitter of aequorin (excited BFP) to the green fluorescent protein (Johnson *et al.*, 1962; Johnson, 1967; Morin and Hastings, 1971b), as represented by the following scheme, wherein "GFP" stands for the green fluorescent protein:

$$\text{aequorin and GFP} \xrightarrow{Ca^{2+}} \text{BFP* and GFP} \longrightarrow \text{BFP and GFP*} \longrightarrow \text{BFP and GFP} + \text{light}$$

In the past few years, green fluorescence similar to that of GFP has been found in photogenic cells of a number of bioluminescent coelenterates, and the fluorescent chromophore, which was found to be actually protein bound in some of the species, was considered to be the *in vivo* light emitter, postulating an energy transfer to this chromophore from the light emitter of a bioluminescence reaction (Hastings and Morin, 1969; Morin and Reynolds, 1969; Morin and Hastings, 1971a,b; Wampler *et al.*, 1972, 1973; Anderson and Cormier, 1973), as in the *Aequorea* bioluminescence. Regarding the bioluminescence reaction preceding the energy transfer to the green fluorescent chromophore, some similarity to the aequorin reaction evidently exists in the chemistry of luminescence at least in *Renilla* and some other species (Cormier *et al.*, 1973; Hori and Cormier, 1973).

The present paper reports the purification and partial characterization of GFP obtained from *Aequorea*, as well as the effects of GFP on the luminescence reaction of aequorin *in vitro*, in an effort to understand and characterize the postulated energy transfer in this species.

Materials and Methods

Aequorin. The material used in this study was extracted and purified from some 30,000 specimens of *Aequorea* essentially according to the method previously reported (Shimomura and Johnson, 1969; Johnson and Shimomura, 1972) except for a new improvement in the present study, namely, the employment of Bio-Gel P-100 (Bio-Rad Laboratories) column chromatography in addition to the previously employed column chromatographies on DEAE-cellulose and Sephadex G-100 (Pharmacia). This procedure is advantageous due to the fact that the behavior of aequorin on Bio-Gel P-100 is considerably different from its behavior on Sephadex G-100 (Kohama *et al.*, 1971). A fraction of the purified aequorin,

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having over 80% purity and containing a practically negligible amount of GFP, was used in this study.

Purification of GFP. In the course of purification of aequorin by DEAE-cellulose column chromatographies, strongly green fluorescent fractions which eluted ahead of the aequorin peak were saved and pooled. GFP from the second DEAE-cellulose column was most abundant. The crude GFP, estimated to contain approximately 10 g of total protein and 500 mg of GFP, was further purified by six steps of column chromatographies, using DEAE-cellulose (Whatman DE-23 and DE-52) and Sephadex G-100 alternately as the packings. In DEAE-cellulose chromatography, the elution was done by linear gradient concentration of NaCl (from 0.02 to 0.3 M) in 10 mM sodium phosphate buffer (pH 6.5) containing 1 mM EDTA; in Sephadex chromatography, 0.3 M NaCl was included in this buffer. Unlike aequorin, GFP behaved similarly on Sephadex G-100 and Bio-Gel P-100, so there was no advantage in using Bio-Gel in addition to Sephadex.

Effluent fractions in each chromatography were measured for absorbance at 280 and 400 nm. The amount of GFP was indicated by the absorption at 400 nm, and the purity of GFP was judged by the ratio of $OD_{400\ nm}$ to $OD_{280\ nm}$. In DEAE-cellulose chromatographies, the elution curves indicated the possible presence of the multiple components of GFP. Complete separation of these components, however, was not attempted by DEAE-cellulose under the conditions involved.

After the six steps of column chromatography, 70 mg of GFP in several fractions each having various ratios of $OD_{400\ nm}/OD_{280\ nm}$ between 0.7 and 0.9 were obtained. These preparations were used in most of the energy-transfer experiments and also where "column-purified GFP" is indicated.

Polyacrylamide Gel Electrophoresis of GFP. Analytical disc electrophoresis was performed with 7% gel at a running pH of 9.5 (Ornstein, 1964; Davis, 1964) at 5°, using Tris-glycine buffer. Preparative electrophoresis was carried out with the same gel–buffer system as used in the analytical electrophoresis on Poly-Prep 100 apparatus (Buchler Instruments). The heights of sample gel, stacking gel and separating gel were 7, 11, and 75 mm respectively, and electrophoresis was done at 220 V (75 mA) at 5°.

Molecular weight estimation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Weber and Osborn, 1969) was carried out on 15% gel, using cytochrome *c*, lysozyme, chymotrypsinogen A, ovalbumin, bovine serum albumin, and γ-globulin as the calibration standards. The mobilities (*M*) were calculated from the migration distance of protein (*P*), the migration distance of Bromophenol Blue (*B*), the gel length before staining (L_1), and the gel length after destaining (L_2), by $M = PL_1/BL_2$.

Determination of $E_{1\ cm}^{1\%}$ of GFP. GFP (4 mg) dissolved in 1 ml of 10 mM sodium phosphate buffer (pH 7.0) was desalted by filtration through a column of Sephadex G-25, fine (1 × 15 cm), which had been thoroughly washed with water in advance. The desalted protein solution was immediately diluted with water to a concentration of approximately 1 mg of protein/ml, then, after recording the absorption spectrum, precisely 1 ml of this solution was evaporated to dryness at 60°. The weight of GFP was obtained after further drying the sample for 2 hr at 110°. The effluent of the Sephadex column collected immediately before the filtration of the protein was processed as a control in the same manner as was the protein.

Amino Acid Analysis. The desalted, dried sample of GFP (1 mg) prepared in the determination of $E_{1\ cm}^{1\%}$ was hydrolyzed with 6 N HCl at 110° for 24–48 hr in an evacuated sealed tube. HCl was removed in a vacuum desiccator containing P_2O_5 and NaOH, then the residue was dissolved in citrate buffer. After addition of 120 nmol each of α-amino-β-guanidinopropionic acid and norleucine as internal standards, the sample was analyzed on a Beckman amino acid analyzer using an accelerated method with spherical resin (Spackman, 1963, 1967).

Tryptophan was determined colorimetrically by procedure N of Spies and Chambers (1949). Glucosamine and galactosamine were determined by the method of Roseman and Daffner (1956), after hydrolyzing GFP in 4 N HCl at 110° for 5 hr.

Spectral Measurements. Absorption spectra were recorded on a Perkin-Elmer Model 202 or Model 402 spectrophotometer. Fluorescence spectra and luminescence spectra were measured with an Aminco-Bowman spectrophotofluorometer. Both the excitation spectra and emission spectra were corrected for light source characteristics through an excitation monochrometer and for photomultiplier characteristics through an emission monochrometer.

In the measurements of bioluminescence spectra of the aequorin–GFP system, care was taken to avoid an excessively fast decay in luminescence intensity by adjusting the amount of added Ca^{2+} to a low level; a possible retardation of luminescence rate by addition of Mg^{2+} (Shimomura *et al.*, 1963; van Leeuwen and Blinks, 1969) was not used because this action of Mg^{2+} is not fully understood. The scanning of the spectrum was done at a speed of 10 nm/sec, and a correction for the small decay was made to the recorded spectrum based on the decay curve determined at 470 or 510 nm.

The bioluminescence spectrum of *Aequorea* photogenic organs was measured as follows. A narrow strip of tissue, 2-mm wide, containing the photogenic organs was cut off from the margin of the umbrella of live specimens. The strip was cut into sections 5 mm in length, and several of these sections were placed in a 1-cm cell with a small amount of sea water, followed by dropwise addition of 1 M KCl to induce luminescence. In our observations, the effect of K^+ in inducing luminescence of an intact organ was much stronger and faster than that of Ca^{2+} or other agents. A similar phenomenon in this same species has been briefly reported by Morin and Hastings (1971b).

Fluorescence Quantum Yields. Quinine dissolved in 1 N H_2SO_4 and fluorescein dissolved in 0.1 N NaOH were chosen as the secondary quantum standards. In measuring the fluorescence quantum yield of GFP excited at 366 nm, GFP in 10 mM sodium phosphate buffer (pH 6.5), and quinine in 1 N H_2SO_4 were so prepared that both solutions had an absorbance of exactly 0.050/cm at 366 nm, then the fluorescence emission spectrum for each solution in a 3-mm cell was recorded on excitation at 366 nm at the same instrument settings. The measurement of fluorescence quantum yield excited at 470 nm was carried out in essentially the same manner, with the GFP solution and with fluorescein in 0.1 N NaOH, both having an absorbance of exactly 0.15/cm at 470 nm. In calculation of the quantum yields, the fluorescence quantum yields of quinine and fluorescein were taken as respectively 0.55 (Melhuish, 1961) and 0.85 (Parker and Rees, 1960).

The fluorescence quantum yield of BFP was measured in the same manner using quinine in 1 N H_2SO_4 as the secondary standard with excitation at 350 nm. The quantum yield of the quinine solution excited at 350 nm was assumed to be 0.55, in accordance with the value for excitation at 366 nm. BFP was prepared from aequorin, dissolved in a buffer solution containing 1 mM EDTA, by adding a minimum amount of 10 mM calcium acetate to complete the luminescence reaction

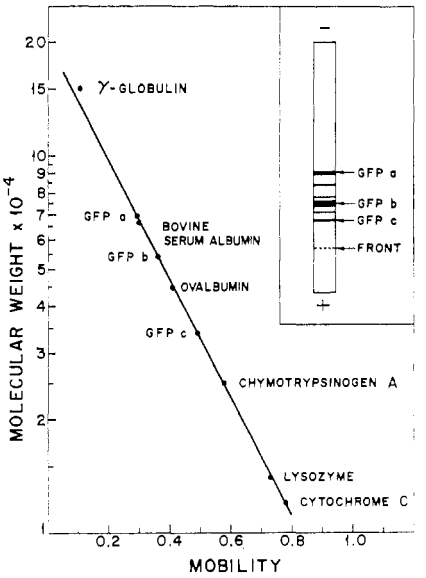

FIGURE 1: Acrylamide gel electrophoresis of the column-purified GFP (upper right) and molecular weight estimation of GFPa, GFPb, and GFPc by sodium dodecyl sulfate acrylamide gel electrophoresis. "Front" indicates the band of Bromophenol Blue.

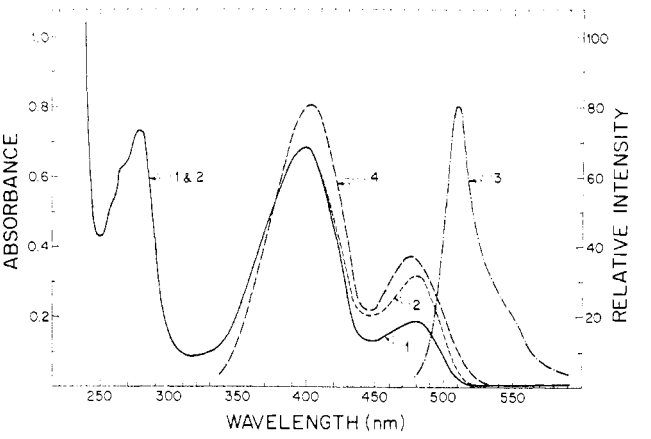

FIGURE 2: Absorption spectra of GFPb (1) and GFPa (2), fluorescence emission spectrum of GFPb excited by 400 nm (3), and fluorescence excitation spectrum of GFPb measured by emission at 508 nm (4). Protein concentrations were 0.85 mg/ml for (1) and (2), and 0.02 mg/ml for (3) and (4), all in 10 mM sodium phosphate buffer (pH 7.0) at 25°. Fluorescence spectra were measured in a 3-mm cell.

in approximately 15 sec, and the product solution was immediately used for the measurement.

Bioluminescence Quantum Yields. The emitted light was measured by an integrating photometer which had been calibrated by *Cypridina* bioluminescence (Johnson *et al.*, 1962; Shimomura and Johnson, 1970b). Quantum yield was calculated on the basis of the emission spectrum of the luminescence system being measured, the luminescence spectrum of the *Cypridina* system, and the sensitivity curve of the photomultiplier.

Preparation of Finely Ground DEAE-cellulose and DEAE-Sephadex. DEAE-cellulose (Whatman DE-52, capacity 1.0 mequiv/g) and DEAE-Sephadex A-50 (Pharmacia, capacity 3.5 mequiv/g) were successively washed with 0.5 N HCl, water, 0.5 N NaOH, water, then finally equilibrated in 10 mM sodium phosphate buffer (pH 7.0), containing 1 mM EDTA. After grinding in a mortar, the material was added to a sufficient volume of the buffer to make a thin suspension and was left standing for 30 min. The sediment was discarded. The material still suspended in the solution was collected by centrifugation, and resuspended in ten volumes of the buffer solution. The suspensions obtained from DEAE-cellulose and DEAE-Sephadex contained solid materials amounting approximately to 1 and 0.4%, respectively, in terms of dry weight.

Dissociation Constant of the Chromophore and the Protein Part of BFP. Aequorin (3.4 mg) was dissolved in 2 ml of pH 7.4 buffer (0.05 M Tris-HCl containing 0.5 mM calcium acetate), and the BFP solution that resulted was washed five times with ethyl ether. The aqueous solution was evacuated to remove dissolved ether, then diluted to 20 ml with the buffer. One milliliter of this solution in a fluorimeter cell was titrated with a 3×10^{-4} M solution of 2-(*p*-hydroxyphenylacetyl)-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine, measuring fluorescence at 465 nm on excitation at 340 nm. The dissociation constant was calculated from the titration curve (*e.g.*, Shimomura *et al.*, 1969) taking 30,000 for the molecular weight of aequorin.

Results

Properties of Purified GFP. Even in a crude preparation, GFP can be easily recognized by its bright green fluorescence and also by the strong absorption peak at 400 nm as well as a weaker absorption peak at 480 nm which overlaps with the fluorescence emission peak of BFP. The fluorescence is so bright that it can be easily recognized in ordinary room light.

GFP purified through six steps of column chromatographies ("column-purified GFP") had ratios of $OD_{400\ nm}/OD_{280\ nm}$ from 0.7 to 0.9 depending on the fractions. Analytical polyacrylamide gel electrophoresis of this material revealed the presence of six green fluorescent components in various amounts (Figure 1). After staining with Aniline Black, all of these six bands were stained. However, no stained band other than these six was found. By means of preparative electrophoresis, the three most abundant components, designated respectively as GFPa, GFPb, and GFPc in the sequence of slower mobility, were separately obtained in electrophoretically pure state from the column-purified GFP for the study of the individual component.

The molecular weights of GFPa, GFPb, and GFPc estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis were 69,000, 53,000, and 34,000, respectively (Figure 1). Neither these values nor the appearance of the stained bands were affected when solutions of the samples in various concentrations were left standing, or even when the samples were treated with urea. Furthermore, analytical gel electrophoresis of a crude extract before any column chromatographic purification showed green fluorescent bands with the same characteristics as indicated in Figure 1. Thus, the multiple components of GFP are evidently genuine and naturally occurring rather than artifacts.

The most abundant of these components was GFPb, which was present in an amount probably close to 50% of the total GFP, and the second most abundant was GFPa. The absorption spectrum of GFPa and GFPb, in the same concentrations of protein, coincided with each other for the region of the peaks at 280 and 400 nm, but differed for the peak at 480 nm (Figure 2). For GFPa and GFPb, the values of $E_{1\ cm}^{1\%}$ for the absorption peaks at 280 and 400 nm were 8.6 and 8.1, respectively, and for both the values of absorbance followed Beer's law. The absorption peaks at 480 nm of both GFPa and GFPb, however, showed an apparent deviation from Beer's law, and the ratios of $OD_{480\ nm}/OD_{400\ nm}$ increased by dilution; thus, at the protein concentrations of 1.1, 0.52,

TABLE I: Amino Acid Composition of GFPb.

	Residues/ 53,000 g of Protein	Nearest Integral No./ 53,000 g	Integral No. × Formula Wt of Residues
Lysine	37.2	37	4,743
Histidine	16.5	17	2,332
Arginine	11.6	12	1,826
Aspartic acid	59.0	59	6,791
Threonine	28.7	29	2,933
Serine	17.3	17	1,481
Glutamic acid	49.9	50	6,457
Proline	23.1	23	2,234
Glycine	44.0	44	2,511
Alanine	21.9	22	1,564
Valine	32.2	32	3,173
Methionine	9.3	9	1,181
Isoleucine	24.8	25	2,829
Leucine	35.2	35	3,961
Tyrosine	18.8	19	3,139
Phenylalanine	22.9	23	3,385
Tryptophan	0.0	0	0
Half-cystine	5.7	6	619
Total			51,159

0.26, and 0.12 mg per ml, the ratios for GFPa solutions were respectively 0.43, 0.48, 0.50, and 0.52, whereas the ratios for GFPb solutions were respectively 0.26, 0.32, 0.35, and 0.37.

The fluorescence emission spectrum of GFPa was identical with that of GFPb (λ_{max} 508–509 nm, shoulder at 545 nm) shown in Figure 2, and both spectra were independent of excitation wavelength and also generally in good agreement with the spectrum of a partially purified sample of the green fluorescent protein reported by Cormier *et al.* (1973).

In regard to the fluorescence excitation spectrum, although both GFPb (Figure 2) and GFPa showed two peaks, namely, λ_{max} 400 nm and 475 nm, the ratio of heights of these two peaks was different in the two components, as expected from the absorbances of both proteins at 480 nm shown in Figure 2.

The absorption and fluorescence spectra of GFPc were closely similar to those of GFPb described above. The absorption spectra and fluorescence excitation spectra reported here are distinctly different from those of the green fluorescent chromophore of *Renilla* (λ_{max} 500 nm for both excitation and absorption; Wampler *et al.*, 1971), although the fluorescence emission spectra in both cases are almost identical.

The fluorescence quantum yields of GFPa and GFPb measured at 25° were both 0.78 when excited at 366 nm, and both 0.72 when excited at 470 nm, by the procedure and under the conditions described in the Methods section. The small difference of quantum yield when excited at the two different wavelengths might be due to an absorption in the 470–480-nm region by a nonfluorescent chromophore as suspected from the slight (5-nm) difference between the 475-nm excitation peak and the 480-nm absorption peak, or might be due to an effect caused by the different concentrations of the proteins. However, such a small difference in quantum yield also could be introduced from various sources of experimental error.

The molecular weights of GFPa, GFPb, and GFPc given above suggest that these components might be polymeric molecular forms, comprising tetramers, trimers, and dimers,

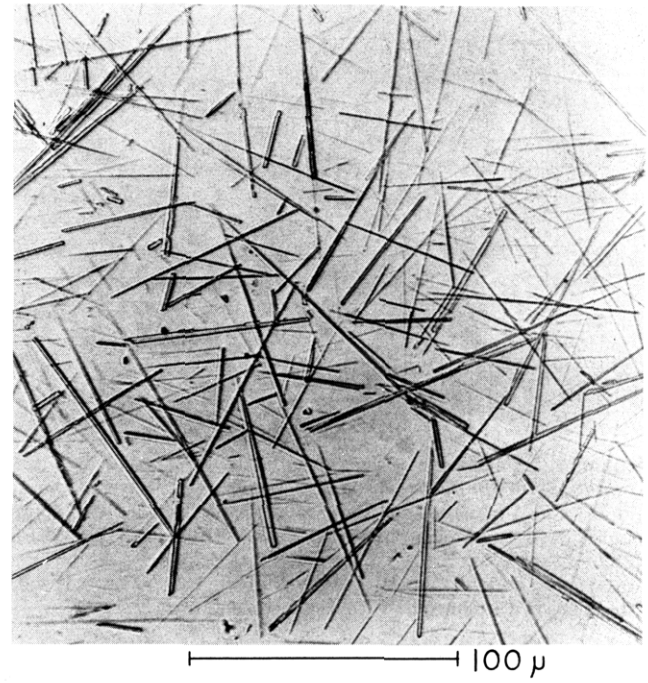

FIGURE 3: Photomicrograph of GFPb crystals.

respectively, of a subunit having a molecular weight of 17,000, although such polymeric forms cannot account for the presence of three other minor GFP components (undesignated in Figure 1). If the polymeric forms actually exist in GFP molecules, as the available evidence suggests, each molecule of monomer (mol wt 17,000) would have to contain a chromophore which absorbs with a 400-nm peak (ϵ 13,800). In regard to the 480-nm absorption peak, further study will be required to interpret the deviation from Beer's law.

The results of amino acid analyses of GFPb are shown in Table I. Neither glucosamine nor galactosamine was a constituent of GFPb.

Crystallization of GFP. When GFPb was dissolved at a concentration of 1 mg or more per ml of 10 mM sodium phosphate buffer (pH 7.0) and was then dialyzed against water at 4°, crystals of fine needles appeared in 20–30 hr. These crystals (Figure 3) could be harvested by centrifugation. Desalting of the GFPb solution with a column of Sephadex G-25, followed by letting the desalted solution stand at 0°, afforded the same crystals. The crystals obtained by either method were easily soluble in 10 mM sodium phosphate buffer, and had properties identical with those of the sample before the crystallization according to various lines of evidence, including absorption spectra and molecular weights by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It follows that the crystals described above comprised crystalline GFPb. Crystallization of GFPa and column-purified GFP were also achieved by the same methods. In the case of column-purified GFP, however, the crystals contained various isomeric components of GFP in the same proportion as found in the material before crystallization although the crystallization efficiently reduced the amount of protein impurities.

Luminescence of Aequorin in the Presence of High Concentrations of GFP. As shown in Figure 4, when aequorin solutions containing a large amount of GFP (1.5–5 mg/ml) were triggered to luminesce by the addition of Ca^{2+}, the intensity at the luminescence peak of aequorin (472 nm) became considerably less than that of aequorin alone, and a new peak appeared, corresponding in position to the fluorescence peak of GFP (509 nm), thus indicating the occurrence

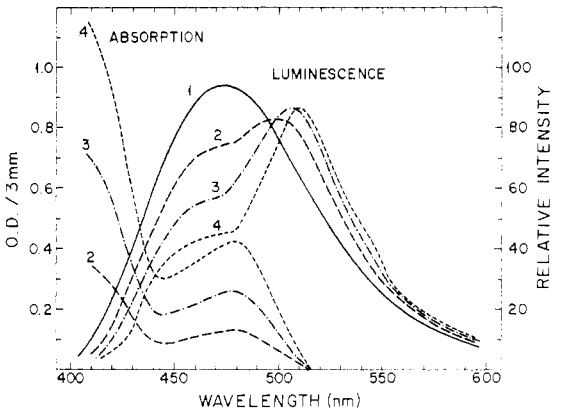

FIGURE 4: Luminescence of aequorin in the presence of high concentrations of GFP. Luminescence reaction was triggered by addition of 2 μl of 10 mM calcium acetate to 20 μl of buffer solution containing 15 μg of aequorin plus: (1) no GFP, (2) 35 μg of GFP, (3) 70 μg of GFP, or (4) 110 μg of GFP. The buffer solution used was 10 mM sodium phosphate containing 1 mM EDTA (pH 7.0). Measured in a 3-mm cell, with slit width of 2 mm, at 25°.

of some kind of energy transfer between the light emitter of aequorin and GFP molecules.

In the region of 410–470 nm, for which the fluorescence emission of GFP, as well as the absorption of BFP (product of aequorin luminescence) have no influence, curves 2, 3, and 4 of Figure 4, especially the last curve, show considerable distortions when compared to the emission curve of aequorin alone (curve 1), indicating a clear involvement of radiative (trivial) energy transfer.

In Figure 4, the intensity relationship between curve 1 and curve 3 was normalized based on the total (integrated) photon yields measured at 470 nm, whereas curves 2 and 4 were inserted by inspection. In the comparison of curve 1 and curve 3 in the 460–470-nm region, only 70% of the difference in the intensities can be attributed to the internal absorption of light by GFP. Thus, although light scattering by the viscous protein solution, and also light reflection due to the small cell as well as to the meniscus which was just at the upper edge of the window, may have made some contribution to the loss of light, some kind of radiationless energy transfer also must be considered a possibility. For the purposes of the present investigation it has seemed unessential to differentiate quantitatively between trivial and radiationless energy transfer, which could obviously be done with appropriate facilities for measuring the lifetime of the donor, *i.e.*, excited BFP.

Luminescence of Aequorin Coadsorbed with GFP. The results of a series of experiments are shown in Figure 5A,B. When luminescence of an aequorin solution is triggered by Ca^{2+} in the presence of a low concentration of GFP (0.15 mg/ml), the emission spectrum indicates very little transfer of energy from the light emitter to GFP (*cf.* curve 1 and curve 2). On the other hand, when a small amount of finely ground DEAE-cellulose or DEAE-Sephadex is first added to the reaction mixture before the addition of Ca^{2+}, the energy transfer becomes notably increased (curve 3). Thus, the spectrum shows an energy transfer to an extent similar to that observed in the presence of a 30-fold concentration of GFP in solution (Figure 4, curve 4). Moreover, in this case, an almost negligible absorption of light by GFP resulted in the undistorted curve for the range of 410–470 nm.

In a separate experiment, a mixture of aequorin with GFP and DEAE-cellulose or DEAE-Sephadex, in a buffer solution containing EDTA, was centrifuged to remove the supernatant which contained a small amount of unadsorbed aequorin and GFP, then the precipitate of the cellulose or the Sephadex which contained coadsorbed aequorin and GFP, almost saturating the anion-exchange groups, was resuspended in a buffer solution, and finally the luminescence reaction was triggered by adding Ca^{2+}. The resulting spectrum (Figure 5, curve 4), which showed an efficient transfer of energy, was practically identical with the bioluminescence spectrum of *Aequorea in vivo* (curve 5).

Interestingly, when aequorin was coadsorbed with FMN (absorption maximum = 450 nm), instead of GFP, a complete transfer of energy from the light emitter of aequorin to FMN was observed (curve 6).

Discussion

As described in the previous section, aequorin solutions containing a high concentration of GFP (3 mg/ml or more) exhibit a noticeable energy transfer from the aequorin light

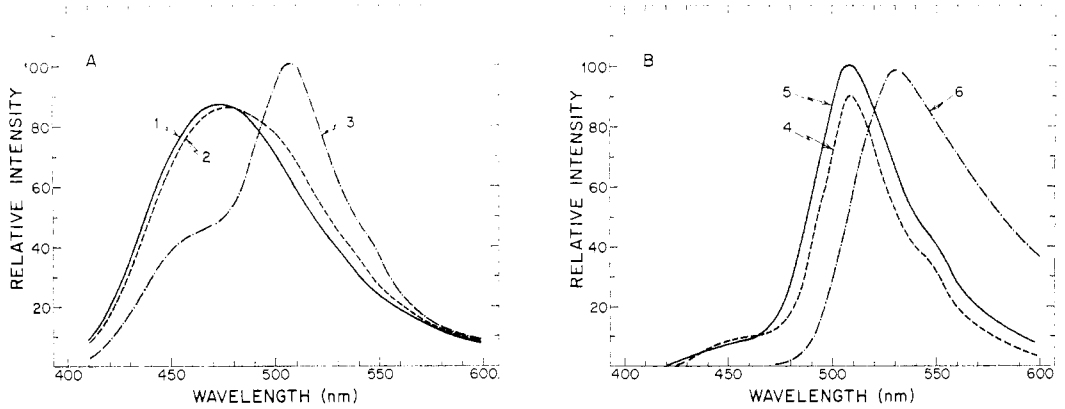

FIGURE 5: Effects of coadsorption of aequorin and GFP on the emission spectrum when triggered by Ca^{2+}. Each luminescent reaction, except (5), was triggered by addition of 30 μl of 10 mM calcium acetate to the following mixtures: (1) 270 μl of buffer solution containing 11 μg of aequorin, (2) 270 μl of buffer solution containing 11 μg of aequorin plus 46 μg of GFP, (3) 270 μl of buffer solution containing 11 μg of aequorin plus 46 μg of GFP plus 100 μl of finely ground DEAE-cellulose or DEAE-Sephadex suspension (see Materials and Methods), (4) 250 μl of buffer solution containing 11 μg of aequorin plus 460 μg of GFP plus 100 μl of the DEAE-cellulose or DEAE-Sephadex suspension was centrifuged, then the precipitate was resuspended in buffer solution making the total to 270 μl, (5) luminescence of photogenic organs of *Aequorea* (see Materials and Methods), (6) the same as (4) except that GFP was replaced with 50 μg of FMN. The buffer solution used was 10 mM sodium phosphate containing 1 mM EDTA (pH 7.0). All measurements were made with a 10-mm cell, with slit width of 2 mm, at 25°.

emitter (BFP*) to GFP when the luminescence reaction is triggered by addition of Ca^{2+}, whereas an aequorin solution containing a small amount of GFP (0.2 mg/ml or less) shows a negligibly small energy transfer. Although the energy transfer observed in the solutions apparently involves a radiative (trivial) transfer mechanism, involvement of radiationless transfer to a smaller extent cannot be excluded (*cf.* Lamola, 1969).

On the other hand, an efficient energy transfer is observed when aequorin and GFP have been coadsorbed on DEAE-cellulose or DEAE-Sephadex prior to the luminescent reaction. In the experiment of Figure 5 (curve 4) about 75% of total protein (350 μg) was adsorbed. The average distance between protein molecules, in the final reaction mixture (300 μl), assuming that the protein molecules were homogeneously dissolved, can be calculated to be approximately 400 Å. In fact, however, the protein molecules were tightly adsorbed on the anion-exchange surface of approximately 1 mg of DEAE-cellulose or 0.4 mg of DEAE-Sephadex. A comparison of the weight of the adsorbents and the total volume of the reaction mixture (300 μl) indicates, considering inaccessibility of the protein molecules to some parts of the adsorbent particles, that actual protein concentration at the surface of the adsorbents should be at least 1000-fold, perhaps several 1000-fold, more than the concentration in the assumed homogeneous solution. Thus, it is postulated that, when the surface of the adsorbent is nearly saturated with aequorin plus GFP, the distance between the protein molecules become short enough to make the mechanism of Förster-type transfer workable (roughly 30 Å), resulting in an efficient energy transfer.

Adsorption of proteins to DEAE materials would be expected to occur at random in regard to many acidic sites of the protein molecule, and, therefore, the relationship between aequorin molecules and GFP molecules on the surface of the adsorbent is also at random. Thus, because of the efficient transfer of energy observed in such a situation, it seems likely that a specific manner of binding, or adsorption, or spatial relationship between aequorin and GFP, such as enzyme–substrate binding, is not necessary in the present instance. This consideration is supported by an efficient energy transfer from the light emitter of aequorin to FMN (*cf.* Figure 5B, curve 6). It can be further inferred that any means which makes the intermolecular distance between aequorin and GFP equal to, or shorter than, the intermolecular distance on DEAE materials, will also result in an efficient transfer of energy.

It follows that, in solution, binding between aequorin and GFP does not occur to a significant extent, under the conditions studied, even in the presence of high concentrations of these proteins. This is in contrast to the case of *Renilla* in which a tight binding between a green fluorescent chromophore and luciferase was reported (Wampler *et al.*, 1971), if we consider the luciferase as equivalent to aequorin in the phenomenon of intermolecular energy transfer. Even so, for the reasons discussed, any binding in any fashion, if it occurs, would be expected in principle to lead to energy transfer.

The quantum yield of Ca^{2+}-triggered luminescence of aequorin in the presence of 3.2 mg/ml of GFP (Figure 4, curve 3) is 0.20, rather than 0.23 of aequorin alone (Shimomura and Johnson, 1969, 1970a); the decrease of quantum yield in this case is understandable on the basis of the involvement of trivial energy-transfer process (emission–absorption–reemission). The quantum yield of Ca^{2+}-triggered luminescence of aequorin coadsorbed on DEAE-Sephadex with GFP (emission spectrum; Figure 5, curve 4) is 0.23, coinciding with that of aequorin alone. Thus, we had expected the fluorescence quantum yield of BFP to be a value close to that of GFP (0.72). Actual measurements, however, gave the values of 0.08 and 0.12, for BFP samples which had contained respectively 0.4 mg/ml of BFP in 10 mM sodium phosphate buffer (pH 7.0) and 0.6 mg/ml of BFP in 10 mM Tris-HCl buffer (pH 7.0) (see Materials and Methods).

We believe this discrepancy should be attributed to an environmental change around the fluorescent chromophore (product chromophore) which takes place after the emission of light. Such a sequence of events could result from two plausible mechanisms, possibly involving both of them simultaneously. In the first, the lifetime of the excited state is shorter than the relaxation time of the protein part of aequorin (see Seliger and Morton, 1968). Consequently, the excited state produced by the chemical reaction contains the product chromophore plus the protein part which has the conformation appropriate to the unreacted chromophore, and light emission takes place before the protein part changes to the conformation appropriate to the product chromophore. The second mechanism involves the partial dissociation of the product chromophore from the specific site of the protein part, after light has been emitted. The product chromophore, 2-(*p*-hydroxyphenylacetyl)amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine, is nonfluorescent in aqueous solution in the absence of the protein part (Shimomura and Johnson, 1973), and the dissociation constant of the product chromophore and the protein part was measured to be 5×10^{-6} M at pH 7.4 at 25°.

An average midsummer specimen of *Aequorea* (diameter 7–8 cm) possesses a total of approximately 200 photogenic organs around the margin of the umbrella, and contains 30–40 μg each of aequorin and GFP, judged by the results of extraction. According to Davenport and Nicol (1955), each photogenic "organ" ($0.4 \times 0.2 \times 0.1$ mm) contains closely packed 6000–7000 photogenic "cells" (average size 10 μ), and each photogenic cell is again densely packed with fine particles (average diameter 0.5 μ). These particles are green under a fluorescence microscope (Morin and Hastings, 1971b), and are possibly equivalent to the "lumisomes" (Anderson and Cormier, 1973) which were found in *Renilla* and several other kinds of bioluminescent coelenterates. Thus, it would be reasonable to consider that these particles are the unit particles responsible for *in vivo* bioluminescence.

Based on the information given above, the content of aequorin and GFP in the photogenic cells can be reasonably estimated to be 5% each or 10% altogether, of the weight of the cells. Although this estimate can vary easily by a factor of two or more, in actual cells, still the extraordinarily high content of the functional proteins distinctly characterizes the cell, and therefore, the subcellular particle as well, and it also suggests that, because of the relatively slight solubilities of aequorin and GFP (2–3%), these proteins are largely undissolved and possibly membrane bound, the latter possibility as previously suggested by Anderson and Cormier (1973) for the lumisomes.

Finally, the content of the functional proteins described above can be plausibly estimated to be two to three times more than that of the proteins in the DEAE materials, which had been initially almost saturated with the proteins and then separated from the supernatant. To have such an efficient packing *in vivo*, the molecules of the functional proteins may

need to be orderly and tightly arranged on a membrane or other structure in the subcellular particles.

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