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Yellow light emission of *Vibrio fischeri* strain Y-1: Purification and characterization of the energy-accepting yellow fluorescent protein*

(luciferase/bioluminescence/energy transfer)

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ABSTRACT **A strain of luminous bacteria, *Vibrio fischeri* Y-1, emits yellow light rather than the blue-green emission typical of other luminous bacteria. The yellow emission has been postulated previously to result from energy transfer from an electronically excited species formed in the bacterial luciferase-catalyzed reaction to a secondary emitter protein, termed the yellow fluorescent protein (YFP). We report here the purification of YFP to homogeneity without loss of the chromophore. The protein was found to be a homodimer of M_r 22,000 subunits with one weakly bound FMN per subunit. The FMN–protein complex was stabilized by 10% (vol/vol) glycerol in the buffers, allowing purification of the active holo-YFP. The protein migrated as a single spot with an isoelectric point of ≈6.5 on two-dimensional polyacrylamide gel electrophoresis and gave an N-terminal sequence of Met-Phe-Lys-Gly-Ile-Val-Glu-Gly-Ile-Gly-Ile-Ile-Glu-Lys-Ile. Addition of purified YFP to a reaction in which luciferase was supplied with $FMNH_2$ (reduced FMN) by a NADH:FMN oxidoreductase resulted in a dramatic enhancement in the intensity of bioluminescence and an additional peak in the emission spectrum at about 534 nm. The resulting bimodal bioluminescence emission spectrum had peaks at 484 nm, apparently due to emission from the luciferase–flavin complex, and at 534 nm, corresponding to the fluorescence emission maximum of YFP. This bimodal spectrum closely matched the emission spectrum *in vivo*.**

Light emission from bioluminescent bacteria is the result of the action of the enzyme bacterial luciferase, a flavin monooxygenase that catalyzes the following reaction:

$$FMNH_2 + O_2 + RCHO \rightarrow h\nu + FMN + H_2O + RCOOH,$$

in which $FMNH_2$ is reduced FMN and RCHO and RCOOH are long-chain fatty aldehydes and acids, respectively (see ref. 1 for a review). Two lines of evidence have suggested that the light emitted comes from the singlet excited state of an enzyme-bound flavin intermediate formed in the luciferase reaction for *Vibrio harveyi*, both *in vivo* and *in vitro*: (*i*) Cline and Hastings found that several mutants of *V. harveyi* having altered luciferase reaction kinetics also had altered bioluminescence emission spectra that had been red-shifted as much as 12–15 nm both *in vivo* and *in vitro* (2, 3), and (*ii*) Mitchell and Hastings showed that the spectrum of light emitted *in vitro* is strongly shifted by alterations in the isoalloxazine nucleus of the flavin (4). It has been suggested that the emitter in the reaction catalyzed by the *V. harveyi* enzyme *in vitro* is a carbon-4a-substituted flavin intermediate, possibly the 4a-hydroxyflavin (5–7).

Some strains of luminous bacteria have emission spectra *in vivo* with λ_{max} shifted relative to the *in vitro* emission spectra because of other proteins in the light-emitting system. Terpstra reported a fluorescent material in crude extracts of *Photobacterium phosphoreum* that stimulated the light reaction *in vitro* (8, 9). *P. phosphoreum* and *Photobacterium leiognathi*, which emit blue light (λ_{max} = 478 nm), do so because of a blue fluorescent protein ("lumazine protein") whose chromophore was identified as 6,7-dimethylribityllumazine (10–14). Addition of lumazine protein to the purified luciferases of *P. phosphoreum* or *P. leiognathi* results in an apparent blue shift in the wavelength maximum of the bioluminescence reaction, an observation that led to the suggestion that the primary excited state in the bacterial bioluminescence reaction might not be the flavin but rather some other species of higher energy (1).

Most strains of *Vibrio fischeri* emit light with a wavelength maximum around 485 nm. However, one strain isolated by Ruby and Nealson (15), designated Y-1, displays an emission spectrum with a peak at 545 nm when grown at temperatures below 22°C, but emits at the usual 485 nm if grown at higher temperatures. The striking difference in the color of emission is shown in Fig. 1. The culture on the left is *P. phosphoreum* (NCMB 844) and the culture on the right is *V. fischeri* strain Y-1 (American Type Culture Collection 33715). In their preliminary study of this system, Ruby and Nealson showed that the luciferase from Y-1, like other bacterial luciferases, catalyzes the emission of blue-green light (λ_{max} ≈ 484 nm) (15). Y-1 was subsequently shown by Leisman and Nealson to contain a yellow fluorescent protein (YFP) that, when added to a Y-1 luciferase reaction with $FMNH_2$ supplied by NADH:FMN oxidoreductase, caused a dramatic increase in the emission of light at 534 nm (17). This paper reports the purification to homogeneity of the yellow fluorescent protein, determination of its physical properties, and the optimum conditions for yellow light emission.

METHODS

V. fischeri strain Y-1 (American Type Culture Collection 33715) was grown in 1.5 liters of NaCl complete medium at 18°C in 2.8-liter Fernbach flasks (18). The cells were grown to an OD_{660} of 1.5, centrifuged at 14,300 × *g* for 30 min, and stored at −20°C. Purification steps were conducted at 4°C. Frozen cells (about 150 g) were thawed and lysed by osmotic shock in 600 ml of 10 mM EDTA/1 mM dithiothreitol, pH 7.0, as described (16). Lysis was usually complete within 30 min. Cell debris was removed by centrifugation at 16,000 × *g* for 20 min, and the supernatant was subjected to ammonium sulfate fractionation. The protein precipitating between 40%

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Abbreviations: YFP, yellow fluorescent protein; Y/B, ratio of intensity of emission at 534 nm to intensity of emission at 484 nm; $FMNH_2$, reduced flavin mononucleotide.

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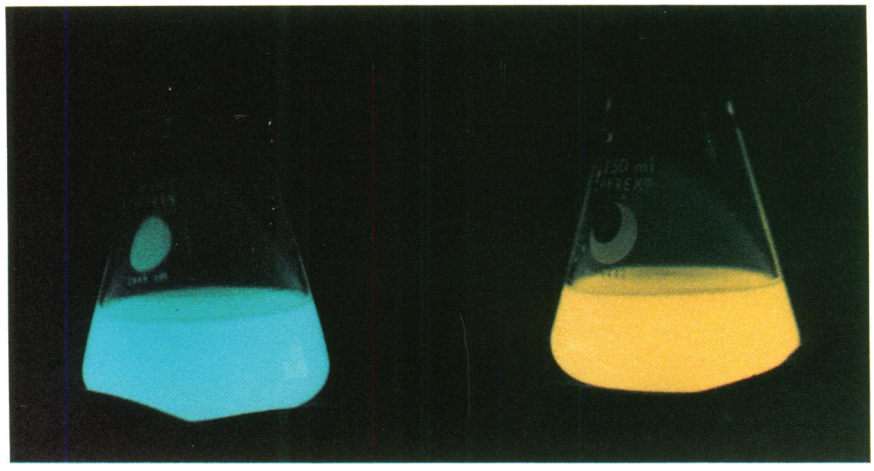

FIG. 1. Cultures of *P. phosphoreum* (*Left*) and *V. fischeri* Y-1 (*Right*). Both cultures were grown at 18°C in sodium chloride complete medium (16). This photograph was taken in the dark without an external light source.

and 70% saturation was resuspended in and dialyzed against 30 mM sodium phosphate/1 mM EDTA/1 mM dithiothreitol, pH 7.0. This step usually resulted in about 200 ml of a solution containing 10–20 mg of protein per ml.

One gram of DEAE-cellulose (DE52, Whatman), previously equilibrated with 30 mM sodium phosphate/1 mM EDTA/1 mM dithiothreitol, pH 7.0, was added per 100 mg of protein, and the suspension was stirred for 45 min. The filtrate (unbound material, including YFP) was concentrated, if necessary, to ≈20 mg per ml by vacuum dialysis. Fifteen milliliters (about 1/10th of the total) was applied to an Ultrogel AcA 54 column (80 cm × 5 cm) and eluted with 30 mM sodium phosphate/1 mM EDTA/1 mM dithiothreitol/2% glycerol, pH 7.0. The YFP fraction, typically 100 ml, was pooled and concentrated to 5 ml by placing it in dialysis tubing with a molecular weight cutoff of 3500 and embedding it in a mound of finely powdered sucrose. Pools from these Ultrogel separations were combined. This sample was dialyzed vs. 30 mM sodium phosphate/1 mM EDTA/1 mM dithiothreitol/10% glycerol, pH 7.0, and applied to a column of DEAE-Sephacel (15 cm × 2.5 cm) previously equilibrated with the same buffer. After loading was complete, a linear gradient was started between the initial buffer and 200 mM sodium phosphate (pH 7.0) (150 ml each); both buffers contained 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Homogeneous YFP was eluted at about 50 mM sodium phosphate. In the early stages of purification, YFP-containing fractions were detected by fluorescence excited with a hand-held mercury vapor lamp. In the later stages of purification, the YFP-containing fractions were located by fluorescence, and the YFP content was determined by absorbance spectra, fluorescence spectra, the coupled bioluminescence assay described below, and $NaDodSO_4$ gel electrophoresis.

Luciferase was prepared from a side fraction of the YFP preparation. The DE52 resin through which the YFP preparation had been filtered was washed once with 0.25 M NaCl/30 mM sodium phosphate/1 mM dithiothreitol/1 mM EDTA, pH 7.0. This resin was filtered and resuspended in 2 M NaCl/30 mM sodium phosphate/1 mM dithiothreitol/1 mM EDTA, pH 7.0, and stirred for 30 min. The resin was filtered, and the filtrate, containing luciferase and NADH:FMN oxidoreductase, was dialyzed vs. 100 mM sodium phosphate/0.5 mM dithiothreitol, pH 7.0. The luciferase was further purified by the affinity chromatography method of Holzman and Baldwin (19).

Absorbance spectra were recorded on a Kontron Uvikon 810 spectrophotometer. Fluorescence and bioluminescence spectra were recorded on an SLM model 8000 spectrofluorometer. Protein determinations were performed with the Bio-Rad protein assay with carbonic anhydrase as a standard. Protein determinations with the Bio-Rad assay were found to agree with values obtained by the Lowry method using the same standard (20) and with values from amino acid analysis of samples that had been hydrolyzed *in vacuo* in 6 M HCl for 24 hr at 110°C. N-terminal sequence analysis was carried out by Edman degradation using a Beckman sequencer; phenylthiohydantoin amino acids were determined by HPLC methods (21).

Luciferase activity was determined by the $FMNH_2$ injection method (16). Bioluminescence emission spectra were recorded from reactions in which $FMNH_2$ was supplied to the luciferase with the NADH:FMN oxidoreductase-coupled assay (16) with the following modifications: total volume was 600 μl in buffer containing 100 mM sodium phosphate, 1% bovine serum albumin, 185 μM NADH, 6 μM FMN, and 0.008% aldehyde (from a 1% sonicated suspension in H_2O). Typically an assay contained 0.8 μM luciferase and was carried out at pH 7.0. Luciferase preparations contained sufficient NADH:FMN oxidoreductase to maintain $FMNH_2$ production. The order of addition was: 1, buffer; 2, aldehyde; 3, luciferase; 4, YFP in Ultrogel column elution buffer (or Ultrogel elution buffer containing no protein); and 5, NADH. NADH was added from a freshly prepared stock of 11.1 mM NADH in 100 μM Tris·HCl (pH 8.0). Total light was recorded with a photomultiplier photometer (22); bioluminescence emission spectra were recorded in the SLM fluorometer by monitoring the emission slit with the excitation lamp turned off. The intensity of bioluminescence emitted at 534 nm was divided by the intensity at 484 nm to give a ratio called the yellow/blue (Y/B) value, allowing comparison of the relative amount of yellow light being produced in different assays.

To determine the identity of the flavin, three experiments were performed. For the first, purified YFP (about 10 nmol of protein-bound flavin) was dialyzed vs. water at 4°C with four changes at 30-min intervals to remove buffer and additives. It was boiled for 15 min to denature the protein and was centrifuged for 5 min at 14,000 × *g* in an Eppendorf Microfuge. The supernatant was lyophilized to dryness and then redissolved in 200 μl of water. This extract was applied to a C_{18} reversed-phase HPLC column by using the system described by Light *et al.* (23). For the second and third experiments, the YFP sample was not dialyzed before denaturation, and each was started with about 3 nmol of protein-bound flavin. In one experiment the flavin-containing extract was exposed to *Naja naja* venom (Sigma) to look for any change in fluorescence properties signifying hydrolysis of FAD to FMN (24). In the other, the flavin-containing extract

was tested as a substrate for the luciferase–FMN oxidoreductase-coupled assay described above.

$NaDodSO_4$/polyacrylamide gel electrophoresis was performed as described by Laemmli (25) on slabs of 12% polyacrylamide. Gels were stained by immersion in 0.05% Coomassie blue R-250 in 10% isopropanol, 10% acetic acid, and destained with 10% isopropanol, 10% acetic acid. Two-dimensional electrophoresis was carried out as described by O'Farrell (26).

RESULTS AND DISCUSSION

Isolation of YFP. YFP was purified by a simple four-step procedure consisting of ammonium sulfate fractionation, DE52 (DEAE-cellulose) batch extraction, Ultrogel AcA 54 chromatography, and DEAE-Sephacel chromatography as described. Purification parameters for a typical preparation are presented in Table 1. YFP purity was estimated by spectral parameters, by the intensity of bands appearing on $NaDodSO_4$ gels, by the intensity and number of spots on two-dimensional gels, and by N-terminal amino acid sequence. Contaminating flavoproteins that interfere with the absorbance spectrum were removed by the DE52 extraction. The DE52 filtrate contained two fluorescent species, YFP and a contaminating fluorophore with an emission λ_{max} at 460 nm; the two were resolved on the Ultrogel column. The pooled YFP-containing fractions from the Ultrogel column had several bands appearing near M_r 20,000–25,000 on $NaDodSO_4$ gels. Of particular interest was a protein that comigrated with YFP on $NaDodSO_4$ gels. It first was detected in a fraction from an Ultrogel column run without a prior DEAE-cellulose extraction step; the column fraction gave a single band on $NaDodSO_4$ gels but was shown by N-terminal amino acid sequence analysis to be heterogeneous, and the contaminating protein was subsequently resolved from YFP on two-dimensional gels of the Ultrogel eluate. After the DE52 batch extraction step was added to the protocol, the second spot no longer appeared on two-dimensional gels of Ultrogel or DEAE-Sephacel eluates. Inclusion of glycerol in the elution and dialysis buffers was essential to prevent dissociation of the fluorophore from the protein during purification, a difficulty that has plagued attempts of others to purify the holoprotein (27). The spectral parameters of the DEAE-Sephacel eluate presented in Table 1 are for the holoprotein; the protein did not lose its yellow color or yellow fluorescence upon dialysis or gel filtration when the buffers contained glycerol.

The most difficult contaminant to remove was a *c*-type cytochrome. After the DE52 batch step, the visible spectra of the protein samples appeared to be mixtures of a flavoprotein and a *c*-type cytochrome. The DE52 filtrate displayed a strong cytochrome *c* spectrum with a small amount of species that absorbed light in the 430–470-nm range. The Ultrogel eluate showed a distinct flavin spectrum (absorbance peaks at 380 and 454 nm) with a small amount of contaminating cytochrome (peaks at 418 and 552 nm), and the DEAE-Sephacel eluate showed a clean flavin spectrum, with no apparent contribution from the cytochrome (Fig. 2). The absorbance maxima are at 382 nm and 454 nm, typical for a flavoprotein. Fig. 3 shows a $NaDodSO_4$/polyacrylamide gel (12%) of typical elution fractions from DEAE-Sephacel.

Table 1. Purification parameters for YFP isolation

Sample	Ratios A_{415}/A_{445}	Ratios A_{280}/A_{445}	Ratios mg/ml*/A_{445}	Protein,* mg
$(NH_4)_2SO_4$ precipitate	1.7	120.4	26.1	6525
DEAE-cellulose filtrate	1.7	37.0	16.3	2445
Ultrogel eluate	2.1	13.6	5.9	528
DEAE-Sephacel eluate	0.7	6.4	2.7	280

Data were compiled from a preparation started with about 150 g of cells.
*Protein concentrations were determined by Bio-Rad assay.

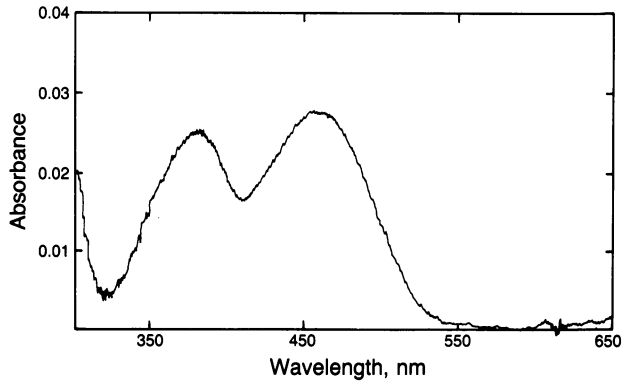

FIG. 2. Absorbance spectrum of the fluorescent fraction eluted from DEAE-Sephacel column. The sample is in 50 mM phosphate/1 mM dithiothreitol/1 mM EDTA/10% glycerol, pH 7.0, and contains ≈30 μg of protein per ml. The spectrum was taken at room temperature.

Properties of YFP. Estimates of molecular weight were made by gel filtration on Ultrogel AcA 54 and by mobility on denaturing polyacrylamide gels. Compared to standard proteins, YFP eluted from the molecular sieve column at a volume appropriate for a protein of M_r 43,600, whereas on denaturing $NaDodSO_4$/polyacrylamide gels, it displayed an apparent M_r of 22,000. Edman degradation of the protein demonstrated a unique N-terminal amino acid sequence (see below). These data suggest that YFP exists as a dimer of identical subunits.

The ligand bound to YFP was clearly a flavin, exhibiting absorbance and fluorescence characteristics of protein-bound flavin. The flavin was shown to be FMN by three methods. First, it was eluted with a retention time identical to that of standard FMN on HPLC (23). Second, it did not display any change in fluorescence properties upon incubation with *N. naja* venom, which would hydrolyze FAD to the more fluorescent FMN (24). Finally, at a concentration of 1.2 μM, it was able to sustain light production in the luciferase–FMN oxidoreductase system without addition of any other source of flavin, ruling out FAD and riboflavin (4). Based on

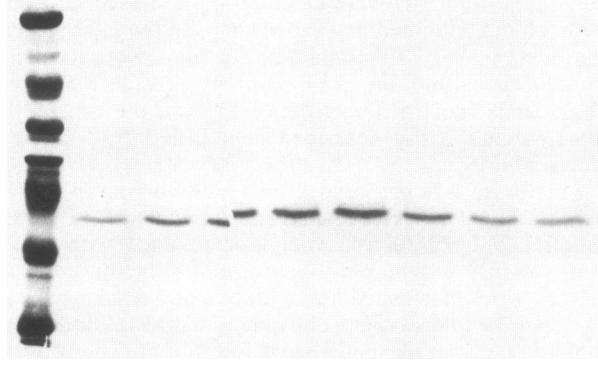

FIG. 3. $NaDodSO_4$/polyacrylamide gel after electrophoresis of consecutive samples of YFP eluted from DEAE-Sephacel column. The lane on the far left contains the standards bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), soybean trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,200). Electrophoresis and staining were carried out as described. The anode is at the bottom of the gel.

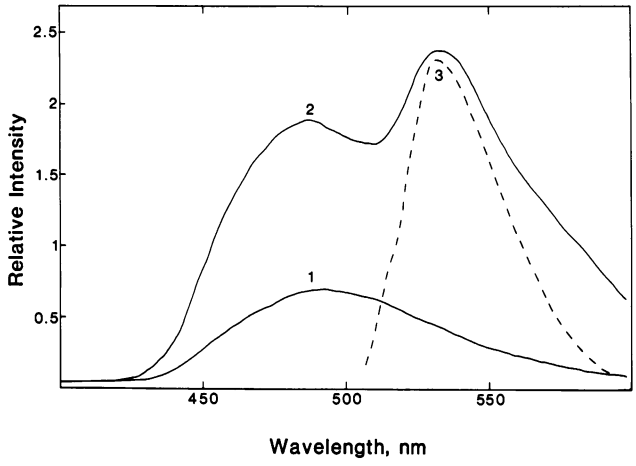

FIG. 4. Bioluminescence assays of luciferase in the presence and absence of YFP. Assays were carried out as described (with 1-decanal as the aldehyde) at 6°C. Curves: 1, spectrum of *V. fischeri* Y-1 luciferase emission with 0.83 μM luciferase; 2, spectrum run with the same amount of luciferase in the presence of 2.5 μM YFP; 3, fluorescence emission spectrum of YFP obtained by irradiating a solution of 2.4 μM YFP in 30 mM sodium phosphate/1 mM dithiothreitol/1 mM EDTA/10% glycerol, pH 7.0, at 380 nm.

the extinction coefficient at 454 nm for YFP [11,600 $M^{-1} \cdot cm^{-1}$, determined by using the method described by Thorpe *et al.* (28)] and protein concentrations determined by the Bio-Rad assay, the DEAE-Sephacel eluate contained 0.77 FMN molecule per 22,000 daltons of protein, suggesting that each subunit of YFP contains one FMN molecule.

Purified YFP was subjected to Edman degradation. The N-terminal sequence was determined to be the following: Met-Phe-Lys-Gly-Ile-Val-Glu-Gly-Ile-Gly-Ile-Ile-Glu-Lys-Ile.

Alteration of Luciferase Emission by YFP. The ability of YFP to alter the color of the light emitted during a luciferase assay is modified by several factors (17). The most striking of these factors is temperature: cells grown above 22°C emit blue-green light and cells grown at 18°C or lower temperatures emit yellow light (15). The emission spectra of luciferase reactions in the presence of various purified protein components of this system are shown in Figs. 4 and 5. All of the bioluminescence spectra in these figures were carried out at the same voltages so that the ordinates are directly comparable. The addition of YFP to a luciferase–FMN oxidoreductase assay resulted in a 3.6-fold increase in the intensity of total light emitted (Fig. 4). The appearance of a new peak at 534 nm shifted the Y/B (534 nm/484 nm) value from 0.64 to 1.26. When YFP was added to the assay mixture in the absence of luciferase, no emission was detected by the fluorometer. Superimposed on the bioluminescence spectra of Fig. 4 is a fluorescence emission spectrum of YFP to demonstrate the identity of the fluorescence emission maximum of YFP with the yellow-shifted bioluminescence maximum.

The effect of YFP on the bioluminescence emission of luciferase is dependent on the relative concentrations of YFP and luciferase (Fig. 5). At lower luciferase concentrations (Fig. 5*A*), the Y/B value varied more upon addition of increasing levels of YFP, as the effect of YFP on intensity of blue emission seemed to be saturated at a concentration lower than its effect on intensity of yellow emission. With a higher luciferase concentration (Fig. 5*B*), both blue and yellow emission increased upon each addition of YFP.

An increase in temperature from 6°C to 21°C resulted in a 2.2-fold increase in the intensity of light emission from a luciferase/YFP reaction. An increase in total light intensity was expected since the total intensity of the reaction catalyzed by luciferase alone increased 1.8-fold with the same increase in temperature. Furthermore, the color shift seen *in vivo* (15) was also seen *in vitro*: in the presence of YFP, the Y/B value at 21°C was 0.61, and the ratio at 6°C was 1.12.

The Y/B value varied with pH as well as with temperature. At 6°C and pH 7.4, the Y/B value was 0.75 and at pH 7.0 it was 0.95; total light intensity at pH 7.4 was 1.5-fold greater than at pH 7.0 (data not shown). Over the greater pH range of 6.2–8.4, the total light intensity increased and reached a peak at about pH 7.4, declining at higher pH values. When assayed in the absence of YFP, the total light intensity of the luciferase reaction is essentially constant between pH 7.0 and pH 7.4 (29); thus some of the increase between 7.0 and 7.4 must be due to the effect of YFP.

CONCLUSION

The mechanism of energy transfer between luciferase and the lumazine protein, extensively studied by Lee and his coworkers, seems to depend on an interaction between the two proteins (30, 31). We have not yet performed experiments to see if a physical interaction between YFP and luciferase can

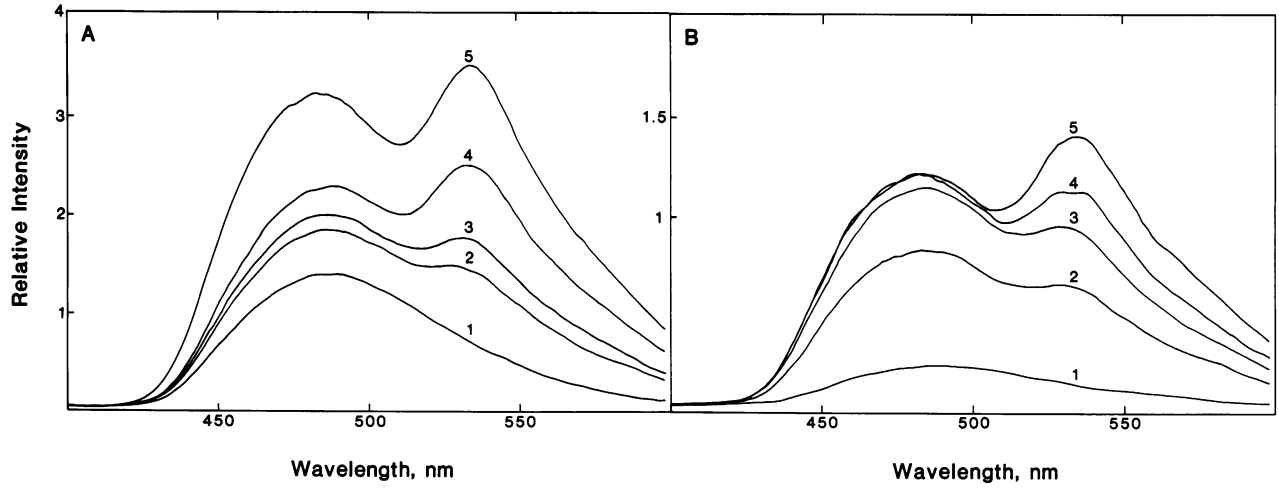

FIG. 5. Effect of luciferase and YFP concentrations on YFP-induced shift of bioluminescence intensity and color. (*A*) Spectra obtained with 0.3 μM luciferase. (*B*) Spectra obtained with 0.8 μM luciferase. All spectra were obtained with the luciferase–NADH:FMN oxidoreductase-coupled assay as described (with 1-dodecanal) at 6°C. Curves in *A* and *B*: 1, luciferase emission in the absence of YFP; 2, luciferase emission with 0.83 μM YFP; 3, with 1.25 μM YFP; 4, with 1.67 μM YFP; 5, with 2.5 μM YFP.

be demonstrated. The two fluorescent proteins are similar in certain respects (subunit molecular weight, ability to enhance luciferase emission), but there is an important difference between them: the lumazine protein effects a transfer leading to emission of light of higher energy than is emitted by luciferase alone (478 nm vs. 490 nm) and YFP effects an energy transfer causing light of lower energy to be emitted (534 nm vs. 484 nm). This difference should allow the two auxiliary proteins to be complementary probes in investigations of the mechanism of energy transfer and the nature of the primary excited species in the bioluminescence reaction. The method reported here for purification of YFP in a stable form establishes the possibility of its use in such studies.

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