Properties of Recombinant Fluorescent Proteins from *Photobacterium leiognathi* and Their Interaction with Luciferase Intermediates†

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ABSTRACT: Ligand binding and luciferase interaction properties of the recombinant protein corresponding to the lumazine protein gene (EMBL X56534) of *Photobacterium leiognathi* have been determined by fluorescence dynamics, circular dichroism, gel filtration, and SDS−PAGE. Scatchard analysis of a fluorescence titration shows that the apoprotein possess one binding site, and at 30 °C the K_ds (μM) are as follows: 6,7-dimethyl-8-ribityllumazine, 0.26; riboflavin, 0.53; and much more weakly bound FMN, 30. All holoproteins are highly fluorescent and have absorption spectra distinct from each other and from the free ligands. The longest wavelength absorption maxima are, respectively (nm, 2 °C), 420, 463, and 458. Ligand binding produces no change in the far-UV circular dichroism; all have mean residual ellipticity at 210 nm of −6500 deg cm^2 dmol^{-1}, the same as the native protein. However, in the bioluminescence reaction only the lumazine holoprotein shows a bioluminescence effect. Fluorescence emission anisotropy decay was used to establish that none of these holoproteins complexed with native luciferase and that the lumazine protein alone formed a 1:1 complex with the luciferase hydroxyflavin fluorescent transient and the luciferase peroxyflavin intermediates, revealed by a dominant channel of anisotropy loss, with rotational correlation time of 2.5 ns, and attributed to excitation transfer from the luciferase flavin donor to the acceptor, the lumazine ligand. The complex stability was sufficient to allow its isolation by FPLC gel filtration and verification by SDS−PAGE. These methods also confirmed the absence of interaction of the holoflavoproteins.

Modulation of the emission spectrum is often observed among bioluminescent organisms. In many of these the molecular mechanism is by an indirect or sensitized process. Chemical energy released at an enzyme active site, a luciferase catalyzed reaction, is transferred to an associated fluorophore that serves as the ultimate light emitter. In *Renilla* and *Aequorea* bioluminescence systems, this fluorophore is the well-characterized "green fluorescence protein" (Ward et al., 1980). A different protein modulates the bioluminescence emission in many types of bioluminescent bacteria. The first of these characterized was called "Lumazine Protein" because the highly fluorescent ligand is 6,7-dimethyl-8-(1′-D-ribityl)lumazine. Certain species of *Photobacterium* are overproducers of lumazine protein. This 21-kDa protein was identified as the bioluminescence emitter because its fluorescence spectrum was the same as the *in vivo* bioluminescence of *Photobacterium phosphoreum*. Also when lumazine protein is included in the *in vitro* reaction with *P. phosphoreum* luciferase and its substrates, $FMNH_2$,[1] O_2, and tetradecanal, the bioluminescence emission spectral maximum at 495 nm is shifted to shorter wavelength and the spectrum is a good match to the fluorescence of lumazine protein, maximum 472 nm [see Lee et al. (1991a) and Lee (1993) for recent reviews].

Photobacterium species emit a blue bioluminescence with type-dependent spectral maxima in the range 472−495 nm. A yellow bioluminescence strain of *Vibrio fischeri* has also been discovered, designated strain Y-1 (Ruby & Nealson, 1977). Although most *V. fischeri* have bioluminescence maxima in the range 490−505 nm, the Y-1 maximum is at 542 nm. From strain Y-1 was isolated a "Yellow Fluorescence Protein", and its primary sequence revealed it to be a close relative of lumazine protein (Baldwin et al., 1990; O'Kane et al., 1991). The yellow fluorescence protein has FMN as a ligand, and the fluorescence spectrum of this bound FMN matches the yellow bioluminescence (Daubner et al., 1987; Daubner & Baldwin, 1989; Macheroux et al., 1987). The mechanism for its modulation of *V. fischeri* bioluminescence is presumably the same as for lumazine protein in *Photobacterium*.

Some strains of *Photobacterium leiognathi* produce in addition to lumazine protein and sometimes in significant excess, a highly fluorescent riboflavin-bound protein (Raibekas & Petushkov, 1990; Lee et al., 1993; Raibekas & Petushkov, 1988). The ratio between lumazine protein and the riboflavin protein in the cells also depends on the phase of growth. Both this riboflavin protein and the yellow fluorescence protein have similar spectral properties, unusual also in that flavoproteins generally are only weakly fluorescent. By a combination of genetic and biochemical experiments, it has been shown that the riboflavin protein and lumazine protein are the same, differing only in the ligand (Illarionov et al., 1994; O'Kane et al., 1994). As in earlier studies of the native lumazine proteins, it was also observed

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[1] Abbreviations: FMN, flavin mononucleotide; LumP, lumazine protein; RFP, riboflavin protein; FMNP, flavin mononucleotide protein; FT, fluorescent transient; Lase, bacterial luciferase; ϕ, rotational correlation time; τ, fluorescence decay lifetime.

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that the ligands in the recombinant protein can be readily interchanged. The recombinant lumazine protein manifests the same bioluminescence properties as the genuine article.

In spite of the presence of the riboflavin protein in these strains of *P. leiognathi*, they still show the same blue bioluminescence spectra, not yellow as might be expected from the fluorescence of riboflavin protein and as exhibited by the strain Y-1 that contain the closely related protein having FMN as its ligand. So the first question to be addressed in this present study is about the role of the ligand, lumazine versus riboflavin, in this bioluminescence modulation. If riboflavin protein does not have a bioluminescence function, then what is its function in the cell?

The bioluminescence effects of lumazine protein with *Photobacterium* luciferases and the FMN-protein in the Y-1 luciferase system are found at protein concentrations below 10 μM. These effects are type specific; lumazine protein for example can blue-shift the bioluminescence with *Vibrio harveyi* luciferase but not at all with *V. fischeri* (Lee, 1982). The yellow fluorescence protein interacts with the Y-1 luciferase, also with *Photobacterium*, but it is without effect in the *V. harveyi* reaction (Daubner & Baldwin, 1989).

It was thought that for these effects to occur at the observed micromolar concentrations luciferase–lumazine protein complexes must be involved, and this idea was supported by fluorescence anisotropy decay measurements (Visser & Lee, 1982). Addition of luciferase to a solution of lumazine protein increased the rotational correlation time of the lumazine protein. Since the luciferase is 77 kDa and is in itself not fluorescent in the visible range, a one to one complex with the lumazine protein should give a lumazine protein mass increased from 21 to about 100 kDa. Complexation was observed between the *P. phosphoreum* lumazine protein and *P. leiognathi* and *V. harveyi* luciferases, but not with *V. fischeri*, consistent with the bioluminescence effects in these cases. However, contradicting the argument, only a very weak complexation was found with the *P. phosphoreum* luciferase, where the bioluminescence is most effective (Visser & Lee, 1982; Lee et al., 1989a,b).

This present work shows that the native luciferase complexation is irrelevant and that the key interaction is one with the luciferase reaction intermediates. This interaction was first detected not by an increase in the rotational correlation time as above but by the finding of a remarkably short time for the loss of fluorescence anisotropy (Lee et al., 1991b). This rapid anisotropy decay was interpreted as due to excitation transfer from the luciferase flavin bioluminescence intermediate to the associated lumazine. It was suggested that this was a feasible mechanism for the bioluminescence modulation.

This present study utilizes the now abundantly available recombinant apoprotein which can be charged by different ligands, the lumazine derivative, riboflavin, and FMN. The fluorescence dynamics properties of these various proteins are measured with increased precision, and it is shown that only with the lumazine bound can the protein associate with the luciferase intermediates. The luciferase intermediate–lumazine protein complex has sufficient stability for isolation by gel filtration chromatography.

MATERIALS AND METHODS

Proteins. The *Escherichia coli* strain BL21 expression host was cultured and the recombinant riboflavin protein extracted and purified as previously described (Illarionov et al., 1994) with some modifications. The cells were grown in Luria–Bertani broth with ampicillin (100 mg/L) at 34 °C to an absorbance of 1.0 at 550 nm. After induction with IPTG (100 mg/L), sucrose (17 g/L) was added, and the cells were grown for a further 5 h at 34 °C and then harvested by centrifugation (10000*g*, 10 min). This and all subsequent steps were done at 4 °C, and the buffer used was 0.05 M P_i (pH 7.4) containing 2-mercaptoethanol (1 mM). The cell cake was dispersed in 6 M urea (3 mL/g wet cells) and disrupted by sonication (3 × 1 min bursts with 2 min cooling intervals). After standing for 4 h the suspension was dialyzed for 8 h against 40 volumes of the buffer containing urea (2 M) and riboflavin (15 mg/L). The urea was then removed by twice dialyzing for 8 h each against the buffer without urea. Cell debris was removed by centrifugation (30000*g*, 1 h). The sample was then loaded on a Q-Sepharose (Pharmacia, Sweden) column (5 × 40 cm) equilibrated with the buffer. The column sizes specified are based on a 10-L cell growth. All column chromatography employed an FPLC system (Pharmacia, Sweden). Gradient elution was made with 1 M NaCl in the buffer, and the eluted fractions were monitored by absorbance at 462 nm. Those fractions containing the riboflavin protein were concentrated (Amicon microflow, YM-10 membrane) and loaded onto Sephacryl (Pharmacia) S-100 HR column (5 × 70 cm), equilibrated with the buffer containing 0.2 M NaCl. All fractions with A_{280}/A_{462} ratio less than 6 were combined and loaded to Q-Sepharose FF (2.5 × 30 cm) equilibrated and eluted with the same buffer containing 0.2 M NaCl. The fractions containing the pure protein were those with the absorbance ratio A_{280}/A_{462} below 4. The final yield was about 250 mg, and the protein homogeneity was greater than 95% by SDS–PAGE. The apoprotein was prepared and other ligands exchanged for the riboflavin by the Amicon filtration procedure in 6 M urea as described by Illarionov et al. (1994).

Recombinant luciferase of the type *P. leiognathi* was purchased from the Institute of Biophysics, Krasnoyarsk, Russia. The bioluminescence specific activity of this luciferase was comparable to our own preparations from *P. leiognathi* strains. It had 95% purity according to SDS–PAGE. For TLC of the separated ligands, the holoprotein in a methanol–chloroform mixture was applied to a cellulose MN 300 polyethyleneimine impregnated plate (Polygram Cell 300 PEI, Brinkmann Instruments). The running solvent system was 4:1:1 butanol/ethanol/water. SDS–PAGE was performed using 15% polyacrylamide gels as described by Laemmli (1970). Four microliters of the protein fraction after gel filtration was loaded on each lane. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

Luciferase Intermediates. To luciferase (0.4 mL, 400 μM) in the buffer at pH 7.0 was rapidly added $FMNH_2$ (0.4 mL, 500 μM). Dodecanol, which stabilizes these luciferase intermediates, was immediately added to a final concentration of 200 μM. All procedures were carried out below 4 °C. The luciferase peroxyflavin was then separated from free FMN by using a column of Sephadex G25, 1 × 8 cm. The 0.5 mL fractions containing the luciferase intermediate were detected by their absorbance spectrum (Lee et al., 1991b). The fluorescent transient (luciferase hydroxyflavin) was prepared by the addition to the luciferase peroxyflavin fraction of 5 μL of tetradecanal saturated in ethanol or

decanal in methanol. The absorbance spectrum was then rerun. The amount of aldehyde was determined by trial and error to be sufficient to convert all the peroxyflavin to fluorescent transient while still maintaining an optically clear solution. The concentrations of fluorescent transient and luciferase peroxyflavin were determined from the absorption spectra after warming and recooling samples to convert them to FMN and luciferase, with the assumption of 1:1 stoichiometry between the FMN and luciferase-bound flavin derivative. After preparation, these intermediates were kept on ice for not more than 2 h before being used in the experiments.

For the chromatography experiments, 200 μL of the appropriate luciferase intermediate sample was loaded to a 1 × 30 cm column of Superose-12 (Pharmacia, Sweden) equilibrated with the buffer, pH 7.0. Proteins were eluted (0.5 mL/min) with this buffer and fractions of 0.5 mL collected. Control experiment, were made with a mixture of luciferase, FMN, decanal saturated in methanol, and the fluorescent proteins, all at the same concentration as in the experiment.

Luciferase concentration were assayed by absorbance assuming ϵ(280 nm) = 85 000 M^{-1} cm^{-1}. For lumazine-, riboflavin-, and FMN-proteins, we used, respectively (M^{-1} cm^{-1}): ϵ(420 nm) = 10 300, ϵ(462 nm) = 12 500, and ϵ-(458 nm) = 12 500.

Steady-State Spectra. Absorption spectra were measured at 23 °C with a Hewlett-Packard 8452 diode array spectrophotometer. Fluorescence and bioluminescence spectra were measured with an SLM (Rochester, NY) model 8000 spectrofluorometer fitted with Glan−Thompson polarizers in the T-configuration. For the collection of fluorescence spectra the polarizers were aligned in the magic angle position. A slit width of 4 nm was used, and samples were contained in a thermostated 3 mL quartz cuvette. For fluorescence spectra of solutions of high optical density, we used 1 mL cuvettes that had a path length of either 1 or 2 mm in the emission direction. All emission spectra were corrected for optical artifacts (e.g., self-absorption) and for the wavelength sensitivity of the instrument, by reference to the absolute emission spectrum of quinine sulfate (Velapoldi & Mielenz, 1980). For the measurement of emission anisotropy, polarizer alignment was first carefully adjusted by reference to the unit polarization for a dilute scattering solution of glycogen and a near zero value for the fluorescence polarization of a dilute riboflavin solution. The total fluorescence emission viewed on the reference arm of the "T", passed first through an interference filter of maximum transmission at 488 nm and color filter (Corning 3-74) to cut down on scattered excitation without introducing fluorescence impurity from the glass itself (e.g., as does the type 3-73).

Circular dichroism in the far UV (190−250 nm) was recorded with a Jasco (Tokyo, Japan) CD spectropolarimeter model J-710. Protein samples in the buffer with 1 mM EDTA, pH 7.0, were maintained at 23 °C while spectra were being recorded. The cuvettes used was a cylindrical quartz cell with a 1 mm path length. The band-pass was 1 nm, time constant 1 s, and step 0.2 nm. Buffer baselines were recorded under identical conditions and subtracted from the data.

Fluorescence Dynamics. Emission decay measurements were made with laser system and single-photon counting electronics described in detail elsewhere (Lee et al., 1989b). The method of data collection and analysis was as adequately detailed elsewhere (Lee et al., 1988, 1989a,b, 1991b), except with a few modifications. The sample was contained in a 3 mL thermostated cuvette, and the path lengths for excitation and emission were 10 mm. Two sides of the cuvette were blackened to eliminate reflected light pulses. The electronic system has been up-dated by using an MCA board (Tennelec-Nucleus, Oak Ridge, TN; PCA-II board) installed in a 486/33 computer for the data collection. Excitation was at 375 nm. The measurement procedure entailed collecting four data sets of decay curves over 512 channels, three with the sample for parallel, perpendicular, and magic angle setting of the polarizers, followed by a dilute glycogen sample to provide the distribution for deconvolution of the laser excitation pulse. The prompt data were collected in the magic angle position and usually timed so that the number of counts in the maximum channel was 4.0 times that in the maximum channel of the sample magic angle data set. It was found that this technique allowed near normalization in the analysis program of the amplitudes of the total fluorescence decay. A single "experiment" on a sample was constituted by these four data sets. Each experiment was subject to the simultaneous analysis procedure (Beechem et al., 1991). The data analysis software (Globals Unlimited, Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL) was also installed on the same computer. When a number of data sets could be combined, by virtue of having the same lifetimes for example, advantage was taken of global analysis techniques to improve the statistical significance of the results.

Most of the experiments were from fluorescing species exhibiting rotation and fluorescence decay on comparable time scales. Therefore "associated" decay models were employed for data fitting where, for example, if two fluorescence intensity decay components (τ) are used, each is assumed to be associated with a species having a distinct rotational correlation time, ϕ. The "goodness of fit" is judged in every case by the statistical parameters, residual and autocorrelation distributions, and χ^2 value, as described elsewhere. For brevity, only the resulting χ^2 is presented in the tables.

RESULTS

Steady-State Spectra. The absorption spectra of the recombinant *P. leiognathi* apoprotein and the holoprotein with the three different ligands are shown in Figures 1 and 2. It is seen that the spectra are different among themselves with maxima as follows (nm): apoprotein, 280; RF protein, 272, 384, 462; FMN protein, 272, 376, 458; lumazine protein, 266, 278, 420. The longer wavelength maxima are red-shifted from the free ligands which are (nm) riboflavin and FMN, 372, 445; the lumazine derivative, 408. The spectra of the corresponding recombinant holoproteins are identical to native lumazine protein and to riboflavin bound to the native lumazine apoprotein from *P. leiognathi* (Illarionov et al., 1994). Although, as will be shown later, FMN is not bound as strongly as riboflavin, at these concentrations there is not sufficient free FMN in this preparation to account for the spectral difference between the FMN protein and riboflavin protein. Therefore, the environment of the bound FMN is distinct from that of the riboflavin. The difference also cannot be attributed to impurities because on separation

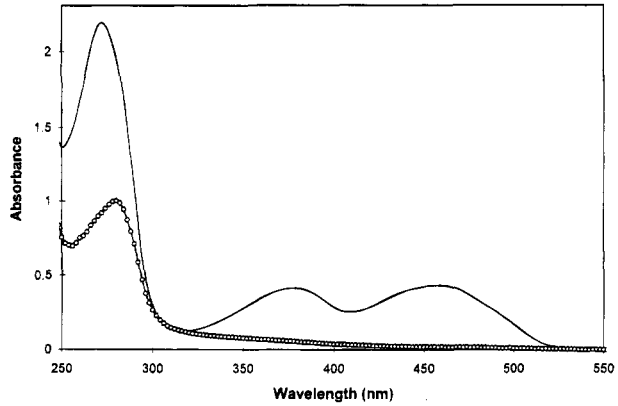

FIGURE 1: Absorption spectra of recombinant apoprotein (open circles) and FMN-bound protein from *P. leiognathi*. All protein concentrations 65 μM; path 1.0 cm, standard buffer, 22 °C.

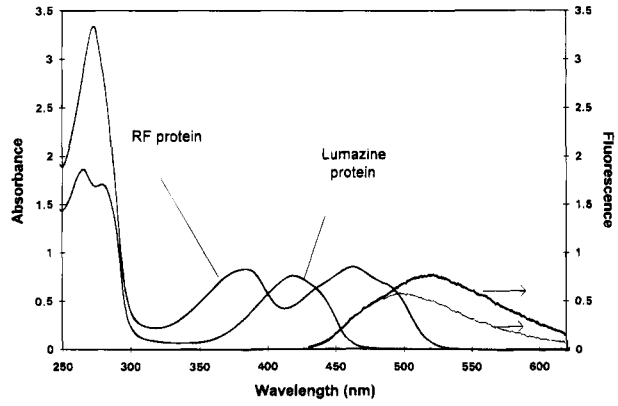

FIGURE 2: Absorption spectra of *P. leiognathi* type recombinant lumazine protein and RF protein. The right-most spectra are the relative fluorescence of a preparation of the luciferase fluorescent transient (heavy line) (0 °C) and the same after subtracting the fluorescence of free FMN (right thin line). Other conditions are as in Figure 1.

of the ligand from each of the three holoproteins, they were assessed by absorption spectrum and TLC as >95% pure.

The protein concentration determined by the Bio-Rad assay was 68 μM. The concentration of bound ligand was estimated using the longest wavelength extinction coefficient (μM): riboflavin, 72; lumazine derivative, 68; and FMN, 35. The 1:1 binding stoichiometry for riboflavin and the lumazine is also found for the native lumazine protein (O'Kane et al., 1985; Kulinski et al., 1987). In the Amicon preparation step for the holoproteins, which is carried out at 0 °C, a considerable amount of FMN was noticed to pass the membrane. Evidently, this FMN binding is a rapid equilibrium which accounts for this apparent binding stoichiometry of only 1:0.5. Gel filtration experiments to be described later lead to the same conclusion.

Temperature Stability. Each holoprotein (20 μM) was held at 100 °C for 5 min and then returned to 0 °C. This resulted in the absorption spectra of the FMN protein and riboflavin protein solutions becoming the same as for the free ligands indicating complete denaturation. In contrast for the lumazine holoprotein the absorption spectrum indicated only about 30% contribution from free ligand. The lumazine evidently helps to refold denaturated protein to its active conformation or protects apoprotein against the temperature denaturation.

Circular Dichroism. Figure 3 shows that the apoprotein and three holoproteins all show the same far-UV circular

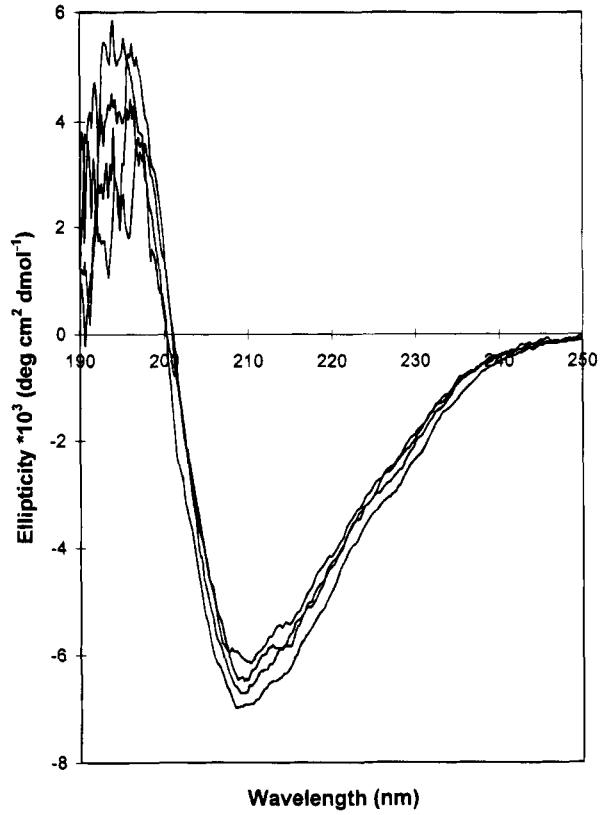

FIGURE 3: Circular dichroism of *P. leiognathi* recombinant apoprotein, lumazine protein, FMN protein, and RF protein. All were measured at 10 μM, 22 °C, 1 mm path. The ordinate is the mean residual ellipticity (186 residues).

dichroism. This indicates that the secondary structure of the protein is not grossly changed on binding any of these ligands. The mean residue ellipticity at the 210 nm minimum is -6.5×10^3 deg cm^2 dmol^{-1}, almost the same as observed for the native protein (Kulinski et al., 1987).

Fluorescence of Holoproteins. Table 1 presents the fluorescence dynamics parameters for each of the ligands free and bound and for the lumazine and riboflavin proteins in the presence of luciferase. For the free lumazine only the fluorescence decay parameters are listed. For the remainder, the total emission decay data are subject to the associated analysis as described under Materials and Methods.

At 0 °C, the fluorescence of the lumazine and riboflavin proteins can be adequately described by two fluorescence decay times and one rotational correlation time. As noted before for the native proteins, the fluorescence decay is almost monoexponential, and the parameters for these two holoproteins are the same as previously measured for the native proteins (Lee et al., 1985, 1993; Illarionov et al., 1994). The τ_1 values 15.3 or 6.0 ns (0 °C), respectively, are significantly longer than for the free lumazine or flavins. Also, the value of correlation time, $\phi \sim 20$ ns (0 °C) indicates that the ligands are rigidly associated in the binding site, because about 20 ns is the value expected from the rotational diffusion of the macromolecule. In Table 1, more than two fluorescence decay times are sometimes fitted, for the purpose of internal comparison.

For the FMN protein even at 0 °C and for all holoproteins at 30 °C, the decay is complex. For an acceptable fit three fluorescence decay times are required and at least two rotational correlation times. Therefore three correlation times are used so that they may be associated with each fluores-

Table 1: Fluorescence Dynamics Properties of Recombinant Holoproteins and Free Ligand[a]

sample	°C	em	α_1	τ_1	α_2	τ_2	α_3	τ_3	r_o	ϕ_1	ϕ_2	ϕ_3	χ^2
Lum	0	460	92	11.2	8	0.64							1.1
LumP	0	460	97	15.3	3	2.2			0.34	21.6	0.1		1.08
LumP	30	460	94	15.1	5	5.2	1	9.7	0.32	9.7	9.7	0.2	1.09
LumP + Lase	0	460	95	15.2	5	5.1			0.31	24.4			1.2
RF	0	520	95	4.98	5	0.1			0.22	0.2			1.2
RFP	0	520	95	6.0	5	3.0			0.22	20.6		0.2	1.05
RFP	30	520	80	5.7	10	3.7	10	4.7	0.22	9.1	9.1	0.15	1.2
RFP	40	520	52	5.6	18	4.0	30	4.5	0.22	7.5	7.5	0.14	1.17
RFP + Lase	0	520	95	6.0	5	5.1			0.22	21.5	21.5		1.05
FMN	0	520	86	4.8	14	0.33			0.22	0.2			1.15
FMNP	0	520	78	6.16	3	4.8	19	4.5	0.23	18.9	18.9	0.2	1.05
FMNP	30	520	29	5.8	71	4.58			0.22	8.4	0.17		1.12

[a] Conditions: 50 mM P_i, pH 7.0; excitation 375 nm; em = emission (nm); α_i = fractional contribution (%); τ_i = fluorescence lifetime (ns); ϕ_i = rotational correlation time (ns); Lum = 6,7-dimethyl-8-ribityllumazine (20 μM); LumP = lumazine protein (16−20 μM); RF = riboflavin; RFP = riboflavin protein (16−18 μM); FMNP = FMN protein (16−18 μM); Lase = *P. leiognathi* rec-luciferase (90 μM).

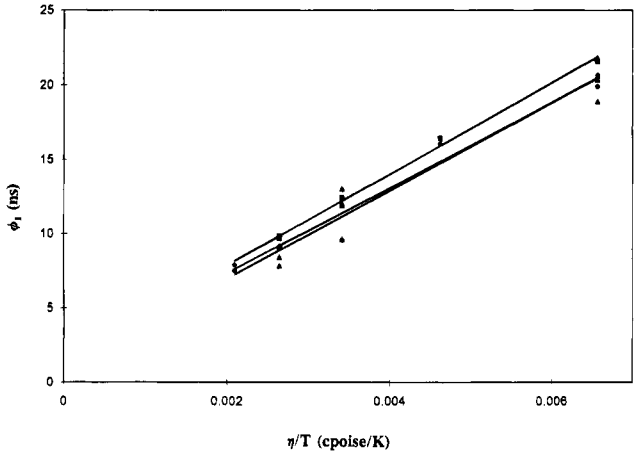

FIGURE 4: Stokes−Einstein plot of the rotational correlation time for the recombinant lumazine protein (triangles), FMN protein (circles), and RF protein (squares).

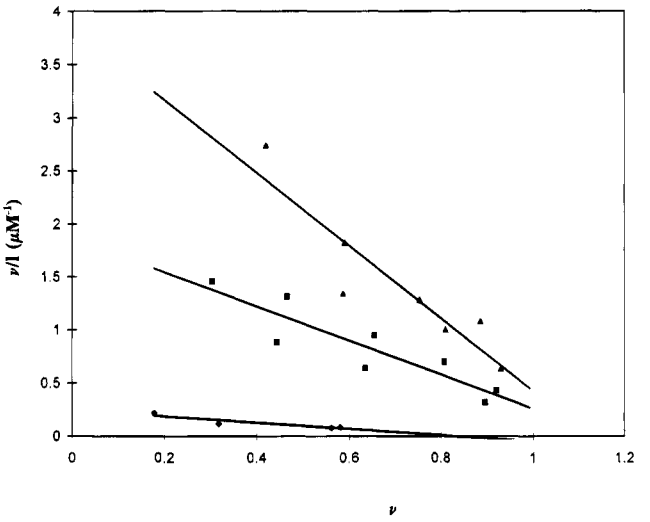

FIGURE 5: Scatchard plot for recombinant RF protein, lumazine protein, and FMN protein. Dissociation constants are recoverered from the ordinate intercept: FMN protein (20 °C) 4.2 μM; RF protein (30 °C) 0.53 μM; lumazine protein (30 °C) 0.26 μM.

cence decay component. For lumazine protein at 30 °C, a value τ_3 = 9.7 ns appears at the 1% level, which would normally be dismissed except that it is near the value for the free lumazine at this temperature (Lee et al., 1993) and is associated with a very fast rotation, ϕ_3 = 0.2 ns. This component, therefore, is attributed to the dissociated free ligand in equilibrium. Similarly for the riboflavin protein and quite clearly at 40 °C, τ_3 = 4.5 ns with ϕ_3 = 0.14 ns is from free riboflavin in equilibrium. For the FMN protein at 30 °C the free ligand is the major species. The second component contributes less than 1% and therefore is not indicated.

Figure 4 shows that the correlation times obey the Stokes−Einstein equation for rotational diffusion of a sphere: $\phi = \eta M_r(v + h)/RT$,where h = hydration. The linearity of the plot indicates that the ligand shows no independent motion at any of these temperatures. For a value of h = 0.4 cm^3/g, the slopes yield M_r = 23 kDa in good correspondence to the value for the native protein.

Table 1 also shows that the fluorescence parameters are not significantly affected in the presence of high concentration of luciferase. In particular, for lumazine protein the ϕ = 24.4 ns is hardly changed from the value without luciferase. This is consistent with the steady-state anisotropy. For a mixture of 5 μM lumazine protein and 40 μM luciferase at 0 °C and 420 nm excitation and 470 nm emission, this anisotropy is 0.21 ± 0.008 compared to lumazine protein alone, 0.208 ± 0.006.

The dynamic fluorescence parameters of the holoproteins were measured at a series of dilutions and the α-parameters used to estimate the concentrations of the free and bound ligand. The equilibrium analysis has been described in detail before (Lee et al., 1992; Lee, 1993). The results are presented in Figure 5 as a Scatchard plot showing that there is close to a single binding site, $\nu \sim 1$, for each ligand. The K_ds recovered are (μM) lumazine protein, 0.26 ± 0.1 (30 °C); riboflavin protein, 0.53 ± 0.2 (30 °C); but significantly higher for the FMN protein, 30 (30 °C) and 4.2 ± 0.9 (20 °C). The results for lumazine and riboflavin proteins are similar to the K_ds of the native *P. leiognathi* protein (Lee et al., 1992).

Luciferase Bioluminescence with the Holoproteins. Figure 2 shows the fluorescence spectrum of a preparation of the luciferase hydroxyflavin fluorescent transient (heavy line). It is a mixture of the fluorescence of the fluorescent transient (right thin line) and some free FMN product, which is the reason the maximum is at a longer wavelength than that for the fluorescent transient itself. The FMN does not contribute below 470 nm, however. The mechanism by which lumazine protein shifts the bioluminescence is not established in detail, but it no doubt depends to some extent on the amount of spectral overlap between the fluorescence of the supposed donor, the fluorescence transient (right thin line), and the

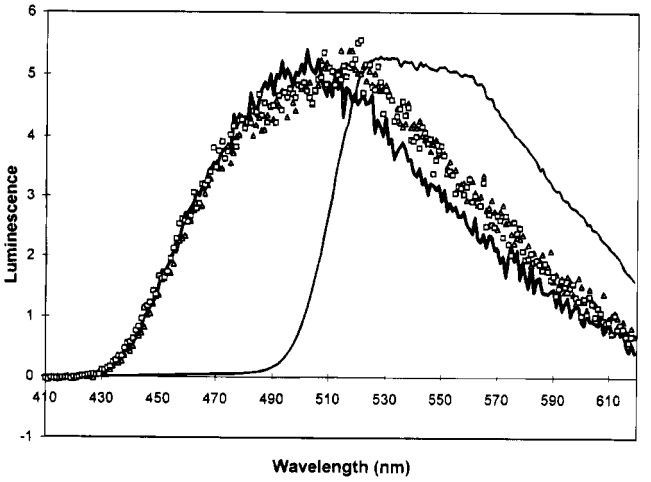

FIGURE 6: Distortion of the bioluminescence spectrum (heavy solid line) on addition of high concentrations of RF protein and FMN protein. (a) 120 μM RF protein (triangles); (b) 100 μM FMN protein (squares); (c) fluorescence spectrum of RF protein (thin solid line) on excitation at 380 nm; all at 2 °C, emission path 2 mm.

absorption of the acceptor, the lumazine protein. For the flavin holoproteins this overlap is very favorable, more than five times higher than for lumazine protein.

The absorption spectrum of the two flavin proteins is also very similar to the yellow fluorescence protein from the Y-1 strain of *V. fischeri*. The flavoproteins have fluorescence maxima however at 530 nm (Figure 6), to be compared with 540 nm for the yellow fluorescence protein (Daubner et al., 1987; Macheroux et al., 1987). Both the yellow fluorescence protein and lumazine protein exhibit an unambiguous bioluminescence effect at the 10 μM concentration levels, but the riboflavin protein or FMN protein at this concentration equally unambiguously have no effect on the *P. leiognathi* luciferase bioluminescence (Illarionov et al., 1994). This observation was also found for the native riboflavin protein (Lee et al., 1993) and when first made several years earlier was an inhibiting factor in its further study.

Figure 6 shows, however, that at a high enough concentration, >100 μM, a shift to longer wavelength bioluminescence is to be seen. These spectra have been corrected for self-absorption, and a thin 2-mm cuvette was used to further minimize corrections. However re-emission of the flavin fluorescence (Figure 6) is probably responsible for this shift but is difficult to factor out. The conclusion is that this long-wavelength shift is an artifact and is not by the same mechanism as that of the yellow fluorescence protein in its reaction.

Interaction with Luciferase Intermediates. Table 2 lists the fluorescence dynamics parameters of the recombinant *P. leiognathi* luciferase bioluminescence intermediates alone and in mixtures with the three holoproteins. The first set is for the fluorescent transient (FT) and the luciferase peroxyflavin (IntII). The parameters are found to be unchanged under these variations in experimental conditions chosen. The luciferase peroxyflavin is only weakly fluorescent so the contribution from the FMN decomposition product is more dominant. The parameters can be assigned simply to the luciferase–flavin intermediate (τ_1) and free FMN (τ_2). Global analysis of the combined data with all parameters linked, but fixing $r_0 = 0.34$ for FT and $\phi_2 = 0.20$ ns for free FMN, gives the same result as for the intermediates from the native luciferase reaction, $\tau_1 = 10.7$ ns, $\phi_1 = 73$ ns (Lee

Table 2: Fluorescence Dynamics Parameters for the *P. leiognathi* Luciferase Intermediates Alone and in the Presence of Fluorescent Proteins[a]

sample concentration	em (nm)	α_1 (%)	τ_1 (ns)	α_2	τ_2	α_3	τ_3	ϕ_1 (ns)	ϕ_2	r_o	χ^2
FT 20 μM	485	90	10.6	10	5			72	0.2	0.39	1.12
FT 30 μM fresh	460	91	10.9	9	5			72	0.2	0.4	1.03
FT 30 μM old	460	95	10.7	5	5			81	0.1	0.36	1.2
FT 10 μM fresh	460	92	10.9	8	5.6			71	0.3	0.35	1.3
FT 12 μM	460	92	10.8	8	5.6			70	0.12	0.33	1.14
FT 28 μM	460	90	10.6	10	2.2			97	10.5	0.37	1.17
IntII 20 μM	460	80	10.4	10	5	10	0.9	66.8	2.5	0.34	1.09
global		95	10.7	5	4.0			72.9	0.2	0.34	1.15
FT 20 μM + glycerol	460	93	10.1	7	1.9			210		0.35	1.10
FT 14 μM LumP 1 μM	460	83	11.2	17	10.8			70.5	10.2	0.35	1.26
FT 14 μM LumP 2μM	460	55	12	45	12			70.9	12.1	0.3	1.26
FT 14 μM LumP 4 μM	460	68	13.1	31	13.1			30	3.8	0.31	1.34
FT 14 μM LumP 8 μM	460	55	13.9	45	13.9			15.4	2.4	0.32	1.2
FT 14 μM LumP 32 μM	460	67	15.1	33	14.5			26.9	4.6	0.32	1.16
FT 14 μM LumP 16 μM	460	66	14.6	34	14.6			21.4	3.9	0.33	1.08
FT 20 μM LumP 17 μM	460	50	14.5	50	14.3			20.3	3.2	0.32	1.1
FT 20 μM LumP 17 μM	460	55	14.6	45	14.4			20.3	2.9	0.32	1.08
same + glycerol	460	50	13.7	50	13.7			35.7	3.2	0.34	1.22
IntII 20 μM LumP 17 μM	460	81	14.4	13	14.4	6	7.7	20.9	2.8	0.32	1.13
IntII 20 μM LumP 17 μM	460	71	14.4	25	14.3	4	4.8	17.5	2	0.32	1.05
IntII 20 μM LumP 17 μM	460	60	14.4	29	14.2	11	4.2	19.6	2.7	0.32	1.13
FT 12 μM RFP 18 μM	460	93	10.7	7	2.7			77	14	0.36	1.12
FT 30 μM RFP 18 μM	460	91	10.8	2	6	8	5	63	20	0.37	1.2
FT 12 μM RFP 18 μM	520	23	10.3	77	5.5			75	17	0.31	1.15
FT 18 μM FMNP 16 μM	460	90	10.6	10	2.7			82		0.33	1.13
FT 12 μM FMNP 16 μM	460	90	10.6	10	2.1			78		0.33	1.10
FT 20 μM FMNP 30 μM	485	70	10.9	20	6.6	10	1.2	79	17	0.38	1.3
FT 18 μM FMNP 16 μM	520	21	10.8	79	5.5			75	15	0.25	1.15
FT 12 μM FMNP 16 μM	520	20	10	80	5.6			75	16	0.32	1.17
FT 20 μM FMNP 30 μM	520	7	10.9	73	6.5	20	4.8	75	12	0.38	1.3

[a] All experiments were made at 2 °C, excitation at 375 nm, in 0.05 M P_i, pH 7.0. Em (nm) = emission wavelength in nm; FT = luciferase hydroxyflavin fluorescent transient; IntII = luciferase peroxyflavin; global = global analysis of previous group of experiments.

et al., 1991b). The present results are more accurate and extensive, however, so this agreement is gratifying. Rigorous error analysis was performed on the global set. At the 67% confidence interval, the range of ϕ_1 is 66−80 ns. Because of its small amplitude (α_2 = 5%), the range of ϕ_2 is very large. In fact the data can be equally well fitted to a single correlation time. In the last line glycerol (30% v/v) is included in the buffer to increase the viscosity 2.9 times, and the ϕ_1 increases accordingly 2.5 times.

The second group of experiments in Table 2 shows the effect of inclusion of different concentrations of lumazine protein with the fluorescent transient. One preparation of fluorescent transient was used for only one or two additions of lumazine protein. Each experiment took about 30 min. During the measurements, which sometimes were made over more than an hour, the concentration of fluorescent transient, initially 14 μM, decreases but not more than 20%. This estimate is from separate measurements of the stability of preparations of the fluorescent transient deduced from the change in the fluorescence signal at 460 nm and formation of FMN in the absorption spectrum. For the emission measured at 460 nm, the FMN contribution is negligible, but it does make itself evident for emission detected at 485 or 520 nm.

For the analysis it was considered that there should be two major fluorescence decay lifetimes, 15.3 and 10.7 ns, corresponding to the two proteins with each associated with a rotational correlation time of that protein. This is not the result. Instead the two lifetime values could not be distinguished (compare Table 1); an average value results which increases toward that of the lumazine protein with increase in its molar ratio. All the parameters depend strongly on the fluorescent transient and lumazine protein molar ratio. Figure 7 shows this in a family of anisotropy decays for the molar ratio changing from 0 to 1 and then from 1 to infinity. It is observed that the fastest decays are in the molar ratio range near unity.

It should be noted that in these experiments we used excitation 370 nm and at an equimolar concentration there is three times more excitation into the fluorescent transient absorption than the lumazine protein. At the extremes of the molar ratio we would expect to see parameters similar to the protein in excess and this is observed (top decay in Figure 7A; second from top in 7B). In the first two lines of the second group (with lumazine protein) in Table 2, the ϕ_1 = 70.5 ns is approximately that of luciferase intermediates in the top group, 72.9 ns. As the concentration of lumazine protein increases, ϕ_1 becomes nearly the same as for lumazine protein. The average value for lumazine protein additions in excess of 16 μM is ϕ_1 = 19.4 ns, and is doubled by the 2.9 times increase in viscosity. Rotation of the protein−protein complex never reveals itself; a ϕ_1 constrained to appropriately high values, >80 ns for the aqueous solution, fails to fit well, $\chi^2 > 1.9$.

It is seen that as the molar ratio approaches equivalence, a second short correlation time, ϕ_2, becomes prominent. From the earlier study with the native proteins, the ϕ_2 = 2.4 ns is attributed to energy transfer in the interaction complex of the two proteins. The average value is 3.1 ns and is not increased significantly by viscosity increase. The approximately equal amplitudes of the two components for FT = 20 μM and lumazine protein, 17 μM, suggest that the

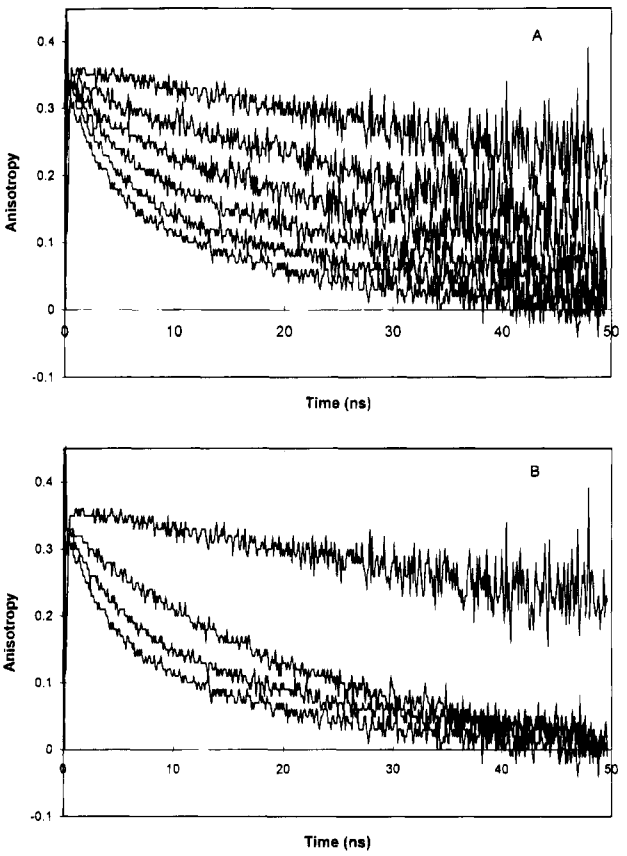

FIGURE 7: Influence of addition of lumazine protein on the fluorescence anisotropy decay of the *P. leiognathi* fluorescent transient (FT = 14 μM). Excitation 375 nm, emission 460 nm, 2 °C, 50 mM P_i, pH 7.0. Lumazine protein from top to bottom: (A) 0, 1, 2, 4, 8, 16 μM; (B) 0, 20 without FT, 32, 20 μM.

dissociation constant for the protein−protein complex should be less than 10 μM.

The third group of experiments in Table 2 shows that an interacting complex exhibiting the energy transfer process also takes place with the luciferase peroxyflavin and lumazine protein. Here the much lower fluorescence efficiency of the luciferase intermediate means that a third fluorescence decay component makes itself evident, arising from the free FMN and filtering through at the 460 nm position for detection. Only the molar ratio of 1:1 is shown, and the results are approximately the same as for the luciferase hydroxyflavin.

No interaction is observed between the luciferase fluorescent transient and either riboflavin protein or the FMN protein. This is shown by the last group of experiments in Table 2. In the first line, detection at 460 nm shows mainly a contribution from unaltered fluorescent transient with a smaller amount from the riboflavin protein. Congruent results are found at longer detection wavelengths and for the mixture with the FMN protein.

All experiments in this last group clearly reveal the two independent protein contributions.

Isolation of Stable Complexes. Figure 8 shows the elution patterns obtained from a variety of mixtures of luciferase and the holoproteins. These patterns are highly reproducible. In the elution pattern line A, it can be seen that there is no evidence for any interaction between unreacted luciferase (fraction 23) and lumazine protein (fraction 27), in agreement with the fluorescence result. The peak at fraction 36 is the unreacted FMN. Line A should be compared with elution

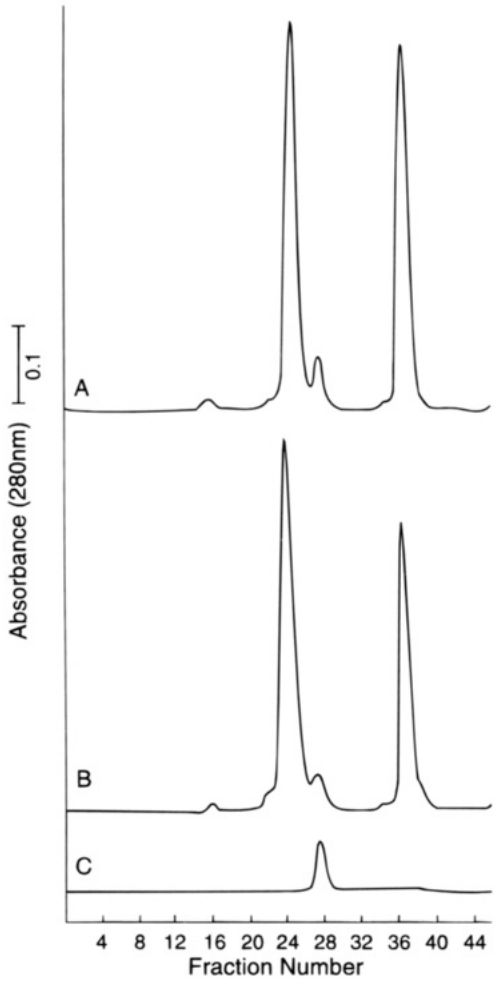

FIGURE 8: (a, top) Chromatography of mixtures of luciferase, luciferase peroxyflavin, and lumazine protein, RF protein, and FMN protein by Superose 12 column chromatography. Total loaded volume, 200 μL; 0.5 mL fractions; absorbance scale at 280 nm, 0.1 AU; 4 °C. (A) 100 μM luciferase, 30 μM lumazine protein, 150 μM FMN. (B) 30 μM luciferase peroxyflavin, 30 μM lumazine protein. (C) 30 μM lumazine protein. (b, bottom) (D) 30 μM luciferase peroxyflavin, 30 μM RF protein. (E) 30 μM RF protein. (F) 30 μM FMN protein.

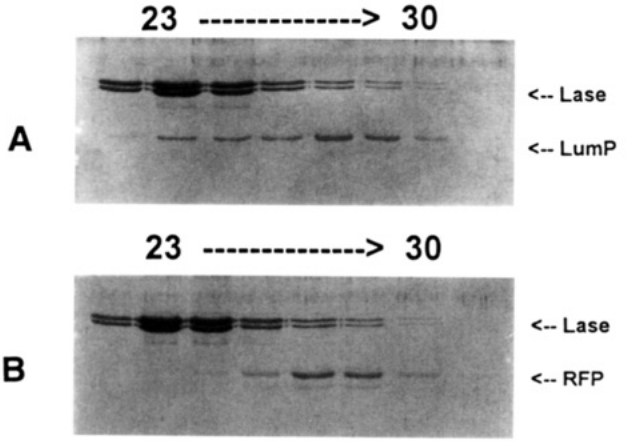

FIGURE 9: SDS−PAGE (15% polyacrylamide) of fractions from Figure 8. Lase: α and β subunits of bacterial luciferase. LumP: lumazine protein, RFP-riboflavin protein. (A) line B; (B) line D.

line B, where $FMNH_2$ is used to generate the luciferase peroxyflavin. The lumazine protein peak is about 30% depleted suggesting an association with the peak fraction 23. Line C is for the same loading of lumazine protein alone. Inclusion of tetradecanal in the reaction mixture to generate the fluorescent luciferase hydroxyflavin produced the same elution pattern with lumazine protein as line B.

Verification of the lumazine protein being associated with the luciferase intermediate fraction, was obtained by SDS−PAGE (Figure 9A). This shows the fractions from line B and reveals the presence (no. 24−25) of the 21-kDa lumazine protein band along with the doublet, the $\alpha-\beta$ dimer subunits (41 and 38 kDa) of luciferase. As a control and evidenced by the result in line A of Figure 8, a mixture of luciferase, FMN, aldehyde, and lumazine protein all separated fully on the SDS−PAGE (not shown).

The same negative result by FPLC (Figure 8b, line D) and by SDS−PAGE (Figure 9B) was observed with the riboflavin protein and FMN protein (not shown) and the luciferase intermediates. The riboflavin protein band does not appear until after the luciferase subunits. Also if the fluorescent transient−lumazine protein complex was held at 20 °C for 2 h, full separation to lumazine protein and luciferase was obtained, just like Figure 8, line A. Ten minutes at 20 °C was not enough to affect dissociation of this complex.

The resolution of the column was not enough to separate the 77-kDa luciferase intermediate from the 98-kDa protein−protein complex. However, it was observed that the fluorescence of a fraction close to the luciferase increased after mixing with LumP. After warming all fractions, lumazine protein fluorescence could be detected at a position corresponding to about 100 kDa. It was estimated that about 30% of the lumazine protein was present in this complex after elution and about 70% of the luciferase intermediate free. Under these chromatography conditions, where the dilution on the column is about 5-fold, an estimate of an upper limit on the protein−protein dissociation constant of 10 μM can be made, provided that the dissociation is not rapid.

The elution patterns of the three holoproteins can be compared in Figure 8, lines C, lumazine protein, E, riboflavin protein, and F, for the FMN−protein. The holoproteins at 30 μM were each loaded onto a Superose-12 column at 4 °C. Although the column dilution factor was about 5-fold,

Table 3: Amount (μM/L) of Chromophore and Fluorescent Proteins in Bioluminescent Bacteria Culture[a]

bacteria	Lum	RF	FMN	LumP	RFP	FMNP	luciferase
PL bright	1.8	20.5	<3.6	1.8	18.2	0	18.8
PL very bright	nd	nd	nd	16	6.8	0	33.3
PL dim	nd	20	0	<0.3	2.4	0	2
VF Y1	2.3	<0.2	19.1	3.2	nd	3.6	10

[a] Extracts made at maximum bioluminescence intensity. nd = not determined; PL = *P. leiognathi*; VF Y1 = yellow strain of *V. fischeri*.

the lumazine and riboflavin proteins passed through the column without any loss of ligand, whereas about 90% of the FMN protein dissociated.

Cellular Fluorescent Proteins. To provide a basis for determining the role of these fluorescent proteins in the cell, assay of these chromophores and proteins were made from cultures of different bioluminescent bacteria at their maximum of bioluminescence intensity (Table 3). As previously concluded in *Photobacterium* brighter bioluminescence is associated with a high lumazine protein content (O'Kane & Lee, 1986). In *V. fischeri* Y-1, all flavin is present as FMN. *P. leiognathi* contains more riboflavin protein than lumazine protein, only small amounts of FMN, and more riboflavin than the lumazine derivative.

DISCUSSION

The results of this work account for the bioluminescence modulation property of lumazine protein by the action of two mechanisms. The first necessary process is the ability to form a stable, approximately 1:1 complex with the luciferase reaction intermediates. The second is that within this complex excitation generated from the luciferase reaction can be efficiently deposited onto the lumazine ligand, accounting for the rapid channel of anisotropy loss, reflected by the parameter ϕ_2 in Table 2. The dissociation constant for this complex, below 10 μM, is in the same range as the concentrations of lumazine protein required to shift the *in vitro* bioluminescence spectrum. The amount of complex formed being dependent of the presence of the two proteins simply explains why the bioluminescence effects of both lumazine protein and the yellow fluorescence protein depend on the concentrations, both in vitro and *in vivo*, on the growth temperature in vivo in the case of the Y-1 strain (Cho et al., 1989), the temperature of in vitro reactions (Daubner et al., 1987), and the time into the reaction with the rise and fall of the fluorescent transient concentration (Lee at al., 1989a; Eckstein et al., 1990).

In this complex, excitation transfer competes with the rate of fluorescence emission from the excited luciferase hydroxyflavin. In the *P. phosphoreum* system, the in vivo bioluminescence spectrum, the fluorescence of lumazine protein and the in vitro bioluminescence with sufficient concentration of lumazine protein, are all the same. This implies that the excitation transfer rate is much faster then the donor fluorescence, and a value of <1 ns^{-1} has been suggested (Lee et al., 1991b; Lee, 1993). The fluorescence lifetime of the fluorescent transient from *P. phosphoreum* has been observed to be about the same as *P. leiognathi*, 10 ns, but it is not sufficiently stable for a close fluorescence dynamics study of the protein−protein interaction.

The *in vivo* maxima of different strains of *P. leiognathi* range from 478 to 490 nm and the 478-nm value is the lowest wavelength maximum attainable in the *in vitro* reaction at lumazine protein concentrations that shift the *P. phosphoreum* bioluminescence to 474 nm. Yet the fluorescence spectrum of the *P. leiognathi* lumazine protein is identical to that of *P. phosphoreum*. It is therefore proposed that the *P. leiognathi* bioluminescence is a mixture of the fluorescence transient emission with that of the bound lumazine fluorescence. The rate of excitation transfer is not as high as in the *P. phosphoreum* complex. In other studies of donor−acceptor systems, the rate of fluorescence energy transfer is measured from the decrease in the donor's fluorescence lifetime, but spectral overlap makes this observation impossible in our case. However, this rate may be extracted from the anisotropy decay, but the analysis is not simple and most workers have resorted to modelling techniques (Vandermeer et al., 1993). This involves a number of assumptions; so, for the sake of a preliminary calculation, the rate of excitation transfer in the complex will be equated to $\phi_2 - 1$, i.e., 0.4 ns^{-1}. If, in the complex, the fluorescence rate from the fluorescence transient remains at 0.1 ns^{-1}, then the emission will contain about 20% contribution from this fluorescence. This would produce a bioluminescence spectral maximum at about 480 nm accounting for what is observed from *P. leiognathi*. The next steps in these studies will be to carry out the model calculations in this and also the yellow fluorescence protein complex and to quantitatively account for the bioluminescence spectra.

The lumazine protein from *P. leiognathi* shows no interaction with unreacted luciferase of the same species, paralleling the case of *P. phosphoreum* lumazine protein with the luciferase of that type. Lack of association of the unreacted luciferase can be rationalized as allowing for the free approach of the substrates to the reaction site. Formation of the stable complex following the reaction may be allowed by a conformational change in the luciferase, for which there is indirect evidence (AbouKhair et al., 1985; Eckstein et al., 1990). Previously, the weak complexation in the case of *P. phosphoreum* was a puzzle, because the bioluminescence spectrum in this luciferase reaction was sensitive at micromolar lumazine protein concentration. The puzzle was magnified by the observation of complexes formed with unreacted luciferase from other species, *V. harveyi* and *P. leiognathi*. Because of its availability, the lumazine protein used for these earlier experiments was mostly of the *P. phosphoreum* type, so it can be concluded that, within the same species, the relevant complex is only the one with the luciferase intermediates.

From the theory of the weak dipole−dipole interaction, it is known that the energy transfer rate depends on several factors including the separation of the donor and acceptor, the mutual angle between the donor emission and acceptor absorption dipoles, and the spectral overlap between the donor emission and acceptor absorption. The requirement for proximity—an estimate of 15 Å has been made (Lee et al., 1991b)—explains why the protein−protein complexation is essential. For the *P. leiognathi* holoprotein with riboflavin or FMN (Figure 1), no energy transfer occurs at all, as just mentioned because there is no protein−protein association. The angle could be altered by a mutation in either luciferase or lumazine protein that involved the protein−protein contact region, explaining why the energy transfer rate could differ between the *P. leiognathi* and *P. phosphoreum* complexes.

The spectral overlap is seen in Figure 2 to be much more favorable for the flavoproteins such as the yellow fluorescence protein than for lumazine protein. The lack of association and, therefore, lack of proximity, in the case of the riboflavin protein (Figures 8D and 9b) explains why only the blue bioluminescence is to be found from *P. leiognathi in vivo*, in spite of the often higher content of the riboflavin protein over lumazine protein. Therefore, in order for the FMN-containing yellow fluorescence protein to have its bioluminescence effect also at these micromolar concentrations, protein−protein complexation must be present. This must be an important reason that the primary sequence of the yellow fluorescence protein is more distinct from that of the closely similar *Photobacterium* lumazine proteins (O'Kane et al., 1991, 1994).

The stereochemistry of the sugar alcohol side group has been shown to be critical for the binding interaction with the apoprotein (Lee et al., 1992). If the ribityl tail fits into a binding cleft, then it is not unexpected that the either the negative charge or the size of the 5′-phosphate of the FMN would lead to weaker binding than for riboflavin or the lumazine. There is no evidence of conformation difference among these holoproteins, at least as revealed by circular dichroism. Therefore, in a comparison of the riboflavin to the lumazine, which are both strongly bound, a plausible suggestion is that the benzene group of the riboflavin is a stereochemical factor inhibiting the association with the luciferase intermediate. The relative binding of these three ligands to the yellow fluorescence protein is not known, but if the FMN is also bound more weakly than the others, this would provide an alternative explanation in terms of concentration of the holoprotein for the remarkable bioluminescence spectral shift observed around 20 °C.

The "antenna" function of lumazine protein in *Photobacterium* and the yellow fluorescence protein in the Y-1 strain is quite evident. The riboflavin protein has no role in the bioluminescence, but it is not conceivable that it would be present in such quantity with no metabolic function. *P. leiognathi* appear to accumulate riboflavin, contrasting with *P. phosphoreum*, an overproducer of the lumazine derivative, and *V. fisheri* Y-1, FMN. Also, reduced riboflavin protein does not replace $FMNH_2$ as a bioluminescence substrate. Presumably, although levels of FMN are low, there is sufficient present in *P. leiognathi* for the bioluminescence reaction. The riboflavin protein has a high solubility, about 10-fold more than riboflavin itself. Either this solubilization is its function or it is a storage protein for riboflavin which might otherwise diffuse through the cell walls and be lost.

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