Direct Measurement of Excitation Transfer in the Protein Complex of Bacterial Luciferase Hydroxyflavin and the Associated Yellow Fluorescence Proteins from *Vibrio fischeri* Y1[†]

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ABSTRACT: Time-resolved fluorescence was used to directly measure the energy transfer rate constant in the protein−protein complex involved in the yellow bioluminescence of *Vibrio fischeri*, strain Y1. In this reaction the putative donor is the fluorescent transient intermediate, luciferase hydroxyflavin, which exhibits a major fluorescence lifetime of the bound flavin of 10 ns. On addition of the acceptor, the *V. fischeri* yellow fluorescence protein containing either FMN or riboflavin as ligand, a rapid decay time, 0.25 ns, becomes predominant. The same results are observed using rec-luciferase from *Photobacterium leiognathi* to produce the donor. Because of favorable spectral separation in this system, this rapid decay rate of 4 ns^{-1}, can be directly equated to the energy transfer rate. This rate is ten times higher than the rate previously observed in the *Photobacterium* luciferase hydroxyflavin−lumazine protein, donor−acceptor system, derived from emission anisotropy measurements. This ten-times ratio is close to the ratio of spectral overlaps of the donor fluorescence with the acceptor absorption, between these two systems, so it is concluded that the topology of the protein complexes in both cases, must be very similar. Energy transfer is also monitored by the loss of steady-state fluorescence intensity at 460 nm of the donor, on addition of the acceptor protein. A fluorescence titration indicates that luciferase hydroxyflavin and the yellow protein complex with a 1:1 stoichiometry with a K_d of 0.7 μM (0 °C). These parameters account for the bioluminescence spectral shifting effects observed in these reactions.

Bacterial bioluminescence results from the addition of molecular oxygen to $FMNH_2$ bound to bacterial luciferase, to form luciferase peroxyflavin, followed by reaction of this with an aliphatic long-chain aldehyde such as tetradecanal [for reviews see Lee et al. (1991a) and Baldwin and Ziegler (1992)]. The aldehyde reaction produces a highly fluorescent species dubbed the "fluorescent transient" (FT)[1] (Matheson & Lee, 1983), proposed to be luciferase hydroxyflavin (Kurfürst et al., 1987). Under appropriate solution conditions, both of these intermediates can be stabilized for several hours and therefore have received considerable attention in regard to understanding the mechanism of this reaction.

Both of these intermediates have characteristic dihydroflavin absorption spectra with absorption maxima around 380 nm (Hastings & Gibson, 1963; Kurfürst et al., 1987; Lee et al., 1989a, 1991b), and the luciferase hydroxyflavin is highly fluorescent with an emission maximum near 495 nm (Matheson & Lee, 1983). This fluorescence spectral distribution is the same as the bioluminescence, therefore identifying the bound hydroxyflavin as the origin of the bioluminescence emission. The mechanism by which the chemical free energy of oxidation is transformed into the excitation is still an intriguing question.

Although *in vivo* many species of bioluminescent bacteria show an emission spectral distribution very similar to the luciferase hydroxyflavin fluorescence, many do not. Most species of *Photobacterium* have a bioluminescence maximum at a shorter wavelength, even to 470 nm, and there exists a yellow bioluminescence strain Y1 of *Vibrio fischeri*, shifted to a longer wavelength, with a maximum at 542 nm (Ruby & Nealson, 1977). These spectral shifts are explained by the fact that there is a second protein involved in these bioluminescence systems that has a ligand having the matching fluorescence characteristics. In *Photobacterium* this is a 21 kDa protein called "lumazine protein" because the fluorescent ligand is 6,7-dimethyl-8-ribityllumazine [see Lee (1993) for a review]. The protein from the yellow bioluminescence strain has a highly similar sequence to lumazine protein, but its ligand is FMN or riboflavin (Daubner et al., 1987; Macheroux et al., 1987; O'Kane et al., 1991; O'Kane & Prasher, 1992; Karatani & Hastings, 1993; Petushkov et al., 1995a). These yellow fluorescence proteins, named FMN-Y1protein and Rf-Y1protein, have the same absorption spectrum and are very fluorescent, with spectra corresponding to the observed yellow bioluminescence from the Y1 strain (Petushkov et al., 1995a).

Our interest is in the biophysical mechanism of the energy transduction into the electronic excited states of the various molecules involved. The inclusion of only micromolar

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[1] Abbreviations: PL, *Photobacterium leiognathi*; Y1, *Vibrio fischeri* Y1; FMN, flavin mononucleotide; Rf, riboflavin; FMN-Y1protein, FMN bound yellow fluorescence protein from *V. fischeri* Y1; Rf-Y1protein, riboflavin-bound protein; LumP, lumazine protein; FFP, lumazine protein from PL recharged by riboflavin; FT, fluorescent transient or luciferase hydroxyflavin; τ, fluorescence decay lifetime.

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concentrations of lumazine protein or yellow fluorescence protein in the luciferase reaction mixture, is sufficient to cause a bioluminescence spectral shift. This implies that the luciferase and the fluorescent protein must be associated in a protein−protein complex of some sort. It has been shown that lumazine protein does form a stable complex with the reaction intermediates already mentioned, luciferase peroxyflavin and luciferase hydroxyflavins, but not with the unreacted luciferase (Petushkov et al., 1995b). It can be rationalized that, if the native luciferase were to preform a protein−protein complex, the lumazine protein would cover the active site and therefore hinder the binding of the $FMNH_2$ substrate to the luciferase active site. From measurement of fluorescence anisotropy decay, a model has been proposed that the initially formed excited state of luciferase hydroxyflavin, is able to transfer its excitation to the lumazine group by the weak dipole−dipole interaction mechanism (Lee et al., 1991b). On the simple model for this process to be efficient, the flavin and the lumazine groups must lie within about 1.5 nm.

It is the purpose of this work to show that the yellow fluorescence protein functions with the luciferase intermediates in a similar fashion. There is a significant advantage in using the yellow fluorescence protein in that its fluorescence spectrum is well separated from the donor fluorescence, and this allows the more precise method of time-resolved fluorescence to be used to recover the energy transfer rate constant rather than the more indirect fluorescence anisotropy decay measurement.

It is also shown here that the yellow fluorescence proteins form a 1:1 complex with the luciferase fluorescent transient and that the topology of this complex must be similar to the one formed with lumazine protein. The weak dipole−dipole mechanism is also supported by the observation that the energy transfer rate constants in the protein complexes containing either lumazine protein or the yellow fluorescence protein are in the same ratio as the respective donor−acceptor spectral overlaps.

EXPERIMENTAL PROCEDURES

The yellow fluorescence proteins and luciferase were extracted and purified from *V. fischeri*, strain Y1 (a gift from K. H. Nealson, Great Lakes Research Institute, University of Wisconsin, Milwaukee, WI) as previously described (Petushkov et al., 1995a). The concentrations of FMN-Y1protein and Rf-Y1protein were determined on the basis of the absorbance at the 460 nm maximum, assuming the same extinction coefficient as FMN, $\epsilon(445\text{ nm}) = 12\,500\ M^{-1}\ cm^{-1}$. The concentration of luciferase hydroxyflavin (FT) was determined from absorption spectra of the free FMN produced after warming and recooling the sample, with the assumption of 1:1 stoichiometry between the bound flavin and the protein. Luciferase concentration was assayed by absorbance assuming $\epsilon(280\text{ nm}) = 85\,000\ M^{-1}\ cm^{-1}$.

The recombinant lumazine protein was purified as previously described (Illarionov et al., 1994) and rec-luciferase type *P. leiognathi* was purchased from the Institute of Biophysics, Krasnoyarsk, Russia. The luciferase peroxyflavin was prepared by mixing $FMNH_2$ and luciferase, followed by addition of dodecanol as a stabilizing agent. The luciferase peroxyflavin was separated from free FMN by using a column of Sephadex G25 (1×8 cm). The luciferase hydroxyflavin was prepared by the addition of tetradecanal to the luciferase peroxyflavin (Petushkov et al., 1995b).

Absorption spectra were measured at 0 °C with a Hewlett-Packard 8452 diode array spectrophotometer. Fluorescence and bioluminescence spectra were measured with an SLM (Rochester, NY) model 8000 spectrofluorimeter fitted with Glan−Thompson polarizers in the T-configuration. All emission spectra were measured with a 2-nm slit width, and the sample was contained in a thermostated 10×3 mm cuvette, with the excitation along the 10-mm direction and the polarizers set in the magic-angle position. All spectra were corrected for optical artifacts (e.g., self-absorption) and for the wavelength sensitivity of the instrument (Velapoldi & Mielenz, 1980). Time-resolved fluorescence was also observed with magic-angle setting using a laser system, single-photon counting electronics, and data analysis described in detail elsewhere (Lee et al., 1989b; Petushkov et al., 1995b). The emission slit width was 15 nm.

For analysis of the fluorescence decay curves, the Globals software was employed (Globals Unlimited, Laboratory for Fluorescence Dynamics, University of Illinois, Urbana, IL). The procedures are described in Petushkov et al. (1995b).

RESULTS

Figure 1 shows the bioluminescence spectrum resulting from the reaction of Y1-luciferase peroxyflavin with tetradecanal alone (open circles) or with the inclusion of FMN-Y1protein (filled circles). The 13 μM concentration of FMN-Y1protein is sufficient to cause the maximum emission intensity ratio, 540 nm:460 nm. In Figure 1 it is seen that the contribution at 460 nm is only about 5%. The same result (not shown) is obtained with the luciferase from *P. leiognathi*, or with Y1-luciferase and Rf-Y1protein, except that the contributions at 460 nm are about 10%.

The FMN-Y1protein and the Rf-Y1protein have the same absorption spectrum shown in Figure 1 (full line). The fluorescence spectrum of the luciferase hydroxyflavin is identical to the bioluminescence spectrum (open circles), and the fluorescence spectra of the yellow fluorescence proteins match the yellow bioluminescence (closed circles). Figure 1 also demonstrates the large spectral overlap between the fluorescence of the putative donor for energy transfer (open circles) and the absorption of the acceptor. This contrasts with the properties of the lumazine protein−luciferase peroxyflavin where the overlap is much smaller. More importantly and of significant advantage, the yellow fluorescence proteins have emission spectra well separated from the donor fluorescence. There is only a small contribution of the yellow fluorescence in the 460−480 nm region, so this means that steady-state and time-resolved fluorescence in this region can be used as direct measurements of energy transfer from the donor to the acceptor.

Energy transfer is also often measured by the decrease in the fluorescence quantum yield of a donor on addition of an acceptor with a concomitant increase in acceptor emission. Figure 2 is such a fluorescence intensity titration of the 460 nm emission from the luciferase hydroxyflavin (9 μM) against additions of FMN-Y1protein. An increase of acceptor fluorescence at 540 nm can also be observed (not shown) but is not practical for a titration experiment due to extraneous fluorescence interference.

In Figure 1, the additions are made every 25 s and the fluorescence signal immediately reduces to a lower level

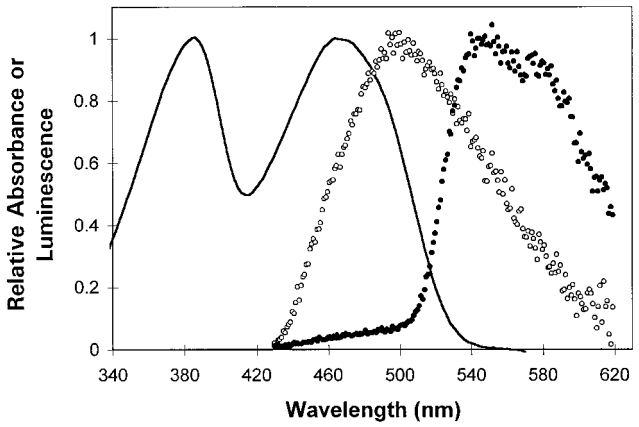

FIGURE 1: Absorption spectra for FMN-Y1protein (solid line) and bioluminescence from the Y1 strain of *V. fischeri* luciferase peroxyflavin (10 μM) on addition of tetradecanal (open circles) and with the inclusion of 13 μM FMN-Y1protein (filled circles); 0 °C, 50 mM P_i buffer, pH 7.0.

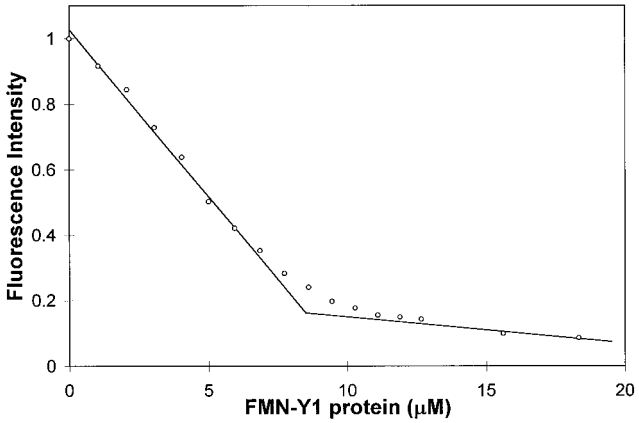

FIGURE 2: Titration of *V. fischeri* FT (initial concentration 9 μM) by FMN-Y1protein. The FT concentration is assayed by 480 nm fluorescence intensity on excitation at 380 nm. A aliquot of 5 μL of FMN-Y1protein was added at 25 s intervals into 0.5 mL total volume to produce a FMN-Y1protein concentration change of 1 μM; 0 °C, 50 mM P_i buffer, pH 7.0.

indicated by each open circle. After about 250 s, i.e., 10 μM total FMN-Y1protein, there is only a small intensity change. There is little change in the luciferase hydroxyflavin concentration over the time taken for the experiment. However, the total absorbance change used, 0.1−0.25 cm^{-1}, causes the fluorescence signal to deviate from a linear dependence on concentration, and the small slope seen after 10 μM is due to this geometrical effect. Neglecting these small corrections (<10%), the titration lines drawn intersect close to a molar ratio of 1:1 for luciferase hydroxyflavin and FMN-Y1protein. Analysis of these data yields a K_d of 0.7 μM (0 °C).

The fluorescence decay curves in Figure 3 show what happens on addition of the yellow fluorescence protein to Y1-luciferase hydroxyflavin (FT). A more detailed analysis is given in Table 1. The luciferase hydroxyflavin alone (curve a) has a major decay lifetime around 11 ns (Table 1, line 1, FTY1). The other types of luciferase hydroxyflavins (e.g., FTPL, *P. leiognathi* luciferase hydroxyflavin, Table 1, line 5) have a similar long lifetime, but for FTY1 the decay is more complex, with a significant contribution ($\alpha_1 = 13\%$) from a 1 ns lifetime. A mixture of Y1-luciferase hydroxyflavin with a slight excess of the FMN-Y1protein, curve d, causes the appearance of a major fast process, 0.25 ns, α_1

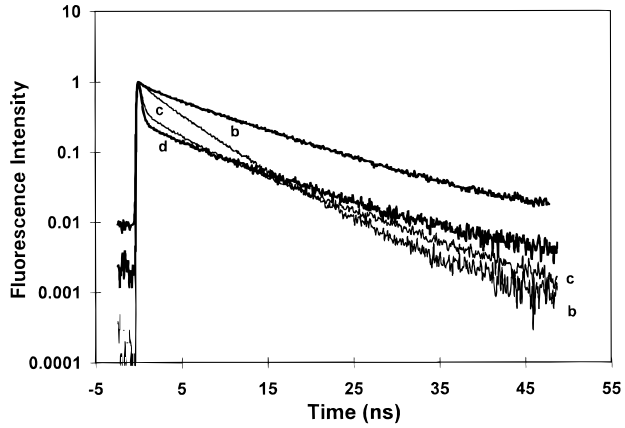

FIGURE 3: Total fluorescence decay. Excitation at 380 nm, 0 °C, 50 mM P_i buffer, pH 7.0. (a) FT (9 μM), 15−30 min after preparation, emission at 460 nm. (b) FT (9 μM), 2 h 30 min after preparation, emission at 480 nm. (c) FT (9 μM) + FMN-Y1protein (13 μM), 3 h 30 min after preparation, emission at 480 nm. (d) FT (9 μM) + FMN-Y1protein (13 μM), 2−20 min after preparation, emission at 480 nm.

= 86% (Table 1, line 4) with a concomitant decrease in the long lifetime contribution, $\alpha_3 = 11\%$.

Obviously this short lifetime for fluorescence decay is due to energy transfer from the luciferase hydroxyflavin donor to FMN-Y1protein acceptor. Even after incubation of the mixture for more than 3h (Figure 3, curve b; Table 1, Sample Age) or increasing the solution viscosity three times to inhibit diffusion (Table 1, Glycerol 20%), the value of the short lifetime component (0.26 ns) remains unchanged and still prominent ($\alpha_3 = 83\%$). By comparing curve b with c, we can see that the luciferase hydroxyflavin alone is more stable in the complex with FMN-Y1protein, i.e., after 3.5 h there still remains the long lifetime component in curve c. The faster decay in curve b over curve c is due to contribution from free FMN, the decomposition product, indicated by the dominant amplitude of the 4.9 ns lifetime (Table 1, second last line, $\alpha_2 = 75\%$) close to that of free FMN.

Quantitative analysis of the fluorescence decay data is in Table 1. The experimental conditions are labeled by the numbers. The measurement sets 1, 5, and 6 use the luciferase hydroxyflavin from *V. fischeri* Y1 (FTY1), and the others use the recombinant *P. leiognathi* luciferase hydroxyflavin (FTPL). In each line, three to eight separate experiments have been combined for the global fitting procedure, with the lifetimes being linked over all experiments. The *P. leiognathi* fluorescent transient result (line 5) is a global fit over 15 experiments from Petushkov et al. (1995b). For internal comparison, each decay curve is fitted by a three-exponential function which also ensures a satisfactory $X^2 <$ 1.3 statistical parameter. In some cases a two-exponential fit is acceptable on the basis of the X^2 test, but most of these (one exception being FTPL) can be rejected because of deviations of residuals and the autocorrelation function (Figure 4). For line 1 however, the $X^2 = 3.5$ for the two-exponential case. Where it is statistically acceptable, a two- or three-exponential fit does not change the final result much. This is shown by comparing measurement sets 2 and 3 for FTPL + Rf-Y1, where both two and three exponentials show the same τ_1 lifetime contribution of about 50%. The value of τ_2 is subject to large uncertainty in all cases. Possibly it arises from some free FMN contributing in the detection spectral region. The second measurement set in Table 1

Table 1: Dynamic Fluorescence Properties of the Luciferase Hydroxyflavin and with the Fluorescent Proteins[a]

sample	em (nm)	α_1 (%)	τ_1 (ns)	α_2 (%)	τ_2 (ns)	α_3 (%)	τ_3 (ns)	χ^2
				1. Change of Ligand				
FTY1	460	13 ± 1	1.0 ± 0.2	15 ± 1	4.9 ± 0.2	72 ± 3	10.9 ± 0.5	1.16
FTY1 + Rf-Y1	460	72 ± 5	0.30 ± 0.03	8 ± 3	4.2 ± 0.8	20 ± 3	12.5 ± 2	1.3
FTY1 + Rf-Y1	480	73 ± 5	0.26 ± 0.01	5 ± 2	2.5 ± 1.5	22 ± 3	10.8 ± 1	1.08
FTY1 + FMN-Y1	480	86 ± 5	0.25 ± 0.01	3.6 ± 0.5	4.2 ± 0.5	11 ± 1	10.5 ± 0.3	1.25
				2. Change of Emission Wavelength				
FTPL	460			5.0 ± 2	4.0 ± 0.8	95 ± 3	10.7 ± 0.3	1.15
FTPL + Rf-Y1	460	52 ± 5	0.32 ± 0.03	15 ± 3	6.7 ± 2	33 ± 3	13 ± 2	1.3
FTPL + Rf-Y1	480	47 ± 5	0.32 ± 0.03	25 ± 3	6.7 ± 2	28 ± 3	13 ± 2	1.3
				3. Two-Exponential Fit				
FTPL + Rf-Y1	460	50 ± 5	0.34 ± 0.03			50 ± 3	10 ± 0.5	1.36
FTPL + Rf-Y1	480	49 ± 5	0.34 ± 0.03			51 ± 3	10 ± 0.5	1.36
				4. Two Fluorescent Proteins				
FTPL + FMN-Y1	480	80 ± 3	0.29 ± 0.01	7 ± 2	3.8 ± 0.3	13 ± 2	10.5 ± 0.3	1.21
FTPL + FFP	470	28 ± 4	0.35 ± 0.1	21 ± 2	3.8 ± 1.2	51 ± 4	11.1 ± 0.3	1.07
				5. Glycerol 20%				
FTY1 + FMN-Y1	480	83 ± 3	0.26 ± 0.01	6 ± 0.8	3.8 ± 0.6	11.0 ± 0.3	9.6 ± 0.4	1.19
				6. Sample Age				
FTY1 + 2.5 h	480	20 ± 3	1.0 ± 0.2	75 ± 3	4.9 ± 0.2	5 ± 1	10.9 ± 0.5	1.16
FTY1 + FMN-Y1 + 3.5 h	480	80 ± 3	0.26 ± 0.01	13 ± 1	4.3 ± 0.5	7 ± 1	10.5 ± 0.3	1.17

[a] All experiments were made at 0 °C, excitation at 380 nm, in 50 mM P_i buffer, pH 7.0. Protein concentration 10−15 μM. Initial ratio of luciferase hydroxyflavin to YFP was close to 1:1; "em" is the detection wavelength. Fluorescence decay was fitted to the equation $I(t) = \sum_i \alpha_i \exp(-t/\tau_i)$. The uncertainties are those recovered by error analysis of the groups of experiments on each line utilizing the Globals rigorous error program (67% confidence interval).

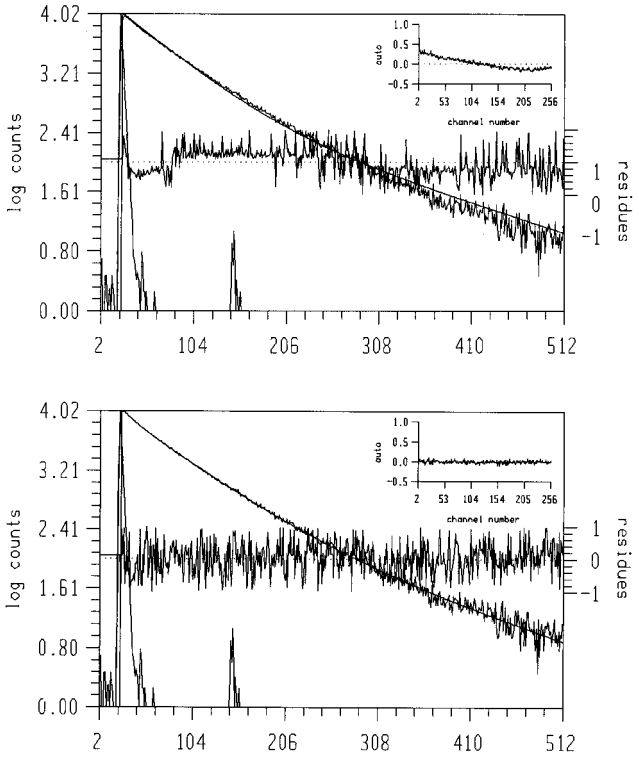

FIGURE 4: Comparison of two-exponential fit (top) with the three-exponential fit for one fluorescence decay experiment contributing to the group in line 1 of Table 1. This is the plot output from the Globals program (0.1 ns/channel). Each panel shows the experimental decay data (noisy line), the fitted function (line), the instrument response function (triangular function around channel 10), and the residuals and the autocorrelation function (top corner). The two-exponential fit is clearly unsatisfactory.

shows that the results do not depend on change in detection wavelength, 460−480 nm.

The first measurement set in Table 1 shows, by comparing the ratio α_1/α_3, that the FMN-Y1protein is more effective than the Rf-Y1protein in inducing energy transfer. This α_1/α_3 ratio reflects the degree of complexation of Y1protein with the luciferase hydroxyflavin. This flavin difference is even more marked for the *P. leiognathi* luciferase hydroxyflavin (Table 1, lines 7 and 10) with the FMN-Y1protein complexed to about the same degree between the two luciferase types (lines 4 and 10). As a control experiment, Table 1, line 11 is the result for *P. leiognathi* luciferase hydroxyflavin with lumazine protein recharged by riboflavin (FFP). Here the decay becomes more complex on addition of this riboflavin-bound protein, with increased minor contributions from some faster process. If this is indeed an energy transfer, the rate cannot be recovered quantitatively due to the increased presence of the acceptor fluorescence at the 470 nm detection wavelength.

DISCUSSION

Progress in elucidating the mechanism of interaction of lumazine protein in the bioluminescence reaction, has been greatly aided by the development of a high expression system for recombinant apo-protein (Illarionov et al., 1994), and the fact that this apo-protein can be charged by different ligands, the lumazine derivative, riboflavin, and FMN (Petushkov et al., 1995b). The primary sequence of the yellow fluorescence protein is close to that of lumazine protein (Baldwin et al., 1990; O'Kane et al., 1991). The yellow bioluminescence Y1 strain of *V. fischeri* can produce the fluorescence protein with either FMN or riboflavin as a bound fluorophore. Both forms are active for shifting the bioluminescence spectral maximum (Daubner et al., 1987; Daubner & Baldwin, 1989; Macheroux et al., 1987; Karatani & Hastings, 1993; Petushkov et al., 1995a). However it was surprising to find that only with the lumazine bound could lumazine protein associate with the luciferase intermediates with a consequent shift of the bioluminescence spectra (Petushkov et al., 1995b).

After mixing with luciferase peroxyflavin, both the FMN-Y1protein and Rf-Y1protein alter the luciferase hydroxyfla-

Table 2. Excited-State Rate Process and Bioluminescence Emission[a]

	fluorescence rate (ns^{-1})	bioluminescence contribution (%)
	Lumazine Protein	
FT* → $h\nu_{490}$	0.1	10
FT* → LumP*	0.4	
LumP* → $h\nu_{470}$	0.066	90
	FMN-Y1protein	
FT* → $h\nu_{495}$	0.1	15
FT* → FMN-Y1*	4.0	
FMN-Y1* → $h\nu_{540}$	0.13	85

[a] Lumazine protein (LumP) 13 μM with *P. leiognathi* luciferase hydroxyflavin (FT, 9 μM), and FMN-Y1protein (13 μM) with Y1 luciferase hydroxyflavin (9 μM). Fluorescence and bioluminescence were measured at 0 °C in 50 mM P_i buffer, pH 7.0.

vin fluorescence property reflecting energy transfer and produce a corresponding shift of the bioluminescence emission. The energy transfer is indicated qualitatively by the enhancement of acceptor fluorescence and more quantitatively by the appearance of the short lifetime in the total fluorescence decay detected at 460−480 nm, close to the maximum emission for the donor. Because of the wide separation of donor and acceptor fluorescence, this fast decay rate can be equated to the rate of energy transfer, 4 ns^{-1}. Although this energy transfer rate is the same for FMN-Y1protein or Rf-Y1protein, the α_1/α_3 amplitude ratio indicates that the Rf-Y1protein is less strongly associated.

In the earlier studies with lumazine protein, the evidence for an energy transfer process came from measurement of emission anisotropy decay. A short correlation time was observed in mixtures with the luciferase intermediates and the energy transfer rate recovered by analysis of this was 0.4 ns^{-1} (Lee et al., 1991b, 1992; Petushkov et al., 1995b). This value is ten times slower than the energy transfer rate to FMN-Y1protein. Table 2 summarizes the values for the rate processes involved and compares the relative contributions of the luciferase hydroxyflavin emission and acceptor protein emission, in the bioluminescence reaction.

For the weakly coupled interaction between singlet−singlet electronic dipolar transitions of a donor and acceptor, the rate of energy transfer is given by the Förster equation:

$$k = AQ_f \kappa^2 \tau^{-1}(R^{-6})J$$

where A is a constant, Q_f is the fluorescence quantum yield of the donor, τ is its fluorescence lifetime in the absence of acceptor, κ is an orientation factor of the two transition dipoles, R is their separation, and J is the spectral overlap of the donor fluorescence with the acceptor absorption. From Figure 1 and by comparison to Figure 3 of Lee et al. (1991b), it can be calculated that the FMN-Y1protein value for J is 7.7 times that for lumazine protein. The observed rate constants are in a similar ratio, 10 times (Table 2).

Table 2 indicates that in the bioluminescence reaction from the FT−FMN-Y1protein complex, the energy transfer rate is 40 times that of the FT fluorescence rate. However in the bioluminescence spectral distribution, the FT fluorescence band accounts for 15% of the total. Based on $K_d = 0.7$ μM, then about 13% of the initial 9 μM FT should be uncomplexed, thus accounting for this bioluminescence result. The observation that, in the lumazine protein system, the contribution of the luciferase hydroxyflavin fluorescence is even less, 10%, in spite of the fact that there is only a 4-fold ratio between the energy transfer and fluorescence rate constants, suggests that these proteins form a more stable complex, as is already reported (Petushkov et al., 1995b). Another factor favoring the lumazine protein emission is that it has a higher quantum yield of fluorescence than the FMN-Y1protein.

It is concluded that the photophysical parameters account for the bioluminescence spectral effects of lumazine and Y1 proteins. Further, lumazine protein and Y1 protein must form a complex with the luciferase hydroxyflavin of similar topology, i.e., of donor−acceptor separation and transition moment orientation, because the energy transfer rate constant ratio is close to the spectral overlap ratio. This idea could be critically tested if the apo-Y1protein can be recharged by flavin analogs with altered spectral properties to change overlap. The lumazine derivative suggests itself as one possibility, although it needs to be cautioned that the ligand itself appears to play some role in the association property. The Rf-Y1protein is not as tightly bound as the FMN-Y1protein, and the lumazine protein has no detectable association with the luciferase intermediates on recharging by flavins (Petushkov et al., 1995b). The development of a high expression system for the Y1 protein will make many systematic studies more practical.

In addition to the spectral effects, the inclusion of lumazine protein or Y1protein, produces bioluminescence kinetic changes, i.e., changes on the seconds timescale, in contrast to nanoseconds as reported here (Cho et al., 1989; Eckstein et al., 1990). On the basis of the protein−protein model, in which the lumazine ligand has to be proximate to the luciferase active site, these kinetic effects can be explained by structural interference between the various partners in the process: the proteins, the long-chain aldehyde, or the aliphatic alcohol such as dodecanol used to stabilize the luciferase intermediates. These results will be reported elsewhere.

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