Electronic Excitation Transfer in the Complex of Lumazine Protein with Bacterial Bioluminescence Intermediates†

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ABSTRACT: Fluorescence dynamics measurements have been made on the bioluminescence reaction intermediates using *Photobacterium leiognathi*, *Vibrio fischeri*, and *Vibrio harveyi* luciferases, both alone and in mixtures with *Photobacterium phosphoreum* lumazine protein. Each luciferase produces a "fluorescent transient" intermediate on reaction with the bioluminescence substrates, $FMNH_2$, tetradecanal, and O_2, and all have a fluorescence quantum yield about 0.3, with a predominant lifetime around 10 ns. The *P. leiognathi* luciferase fluorescent transient has a rotational correlation time of 79 ns at 2 °C, as expected for the rotational diffusion of a 77-kDa macromolecule. In the presence of lumazine protein however a faster correlation time of about 3 ns predominates. This rapid channel of anisotropy loss is attributed to energy transfer from the flavin intermediate bound on the luciferase to the lumazine ligand, reflects the presence of protein–protein complexation, and is greatest in the case of *P. leiognathi*, but not at all for *V. fischeri*. This fact is consistent with the strong influence of lumazine protein on the bioluminescence reaction of *P. leiognathi*, and not at all with *V. fischeri*. The rate of energy transfer is of order 10^9 s^{-1}, much greater than the 10^8 s^{-1} fluorescence rate of the donor. Thus the bioluminescence excitation of lumazine protein could occur by a similar photophysical mechanism of interprotein energy transfer from a chemically excited fluorescent transient donor to the lumazine acceptor.

The migration of electronic excited-state energy is a biologically significant process in many living organisms, photosynthetic ones being the most intensely studied (Holzwarth, 1989). Some bioluminescence systems are also known to carry out electronic energy migration (Ward, 1979). In some types of bioluminescent bacteria, excitation transfer takes place from the chemical reaction site on the bacterial luciferase to another protein which functions as the antenna, the best characterized of these being lumazine protein, named because it contains 6,7-dimethyl-8-ribityllumazine as a reversibly bound ligand (Lee, 1985, 1990; Lee et al., 1991a).

Most types of luminous bacteria are classified in two genera, *Vibrio* and *Photobacterium*. For the *Vibrios* the in vivo bioluminescence spectrum is close to the same as the in vitro bioluminescence from the reaction of their purified bacterial luciferase with $FMNH_2$,[1] tetradecanal, and O_2. For *Photobacterium phosphoreum* bacteria, however, the in vivo bioluminescence has its maximum at 475 nm, in contrast to the in vitro maximum at 496 nm. In *Photobacterium* cells, the bioluminescence emission is known to come from the excited state of lumazine protein. The evidence is that the fluorescence spectrum of lumazine protein has the same maximum at 475 nm and spectral distribution as the in vivo bioluminescence (Gast & Lee, 1978). Further, if lumazine protein is included in the in vitro reaction: (1) the bioluminescence spectral maximum shifts from 496 nm toward 475 nm; (2) the intensity of the bioluminescence and its rate of decay are both increased; and (3) the bioluminescence yield is increased several times (Lee, 1985, 1990; O'Kane & Lee, 1986).

These bioluminescence effects of lumazine protein depend on several factors such as the type of luciferase used and the concentration of lumazine protein (Lee, 1982). For *Photobacterium* luciferases, a concentration of lumazine protein of 10 μM is sufficient to cause these changes, for *V. harveyi* 20–30 μM is needed, and the *Vibrio fischeri* bioluminescence is almost unaffected by lumazine protein concentrations up to 100 μM. For the analysis of the complex fluorescence dynamics data in this work therefore, the *V. fischeri* system will be found to provide a valuable control.

In order for the lumazine protein to exert its effect in the bioluminescence reaction, it has been supposed that lumazine protein and luciferase should be associated in a protein–protein complex, where the lumazine ligand would lie nearby the active site on the luciferase (Lee, 1990). Visser and Lee (1982) detected such complex formation by fluorescence anisotropy decay measurements. They showed that the rotational correlation time (ϕ) of lumazine protein (21 kDa) increased as luciferase (77 kDa) was added to the solution. The outcome of this and a more recent study (Lee et al., 1989b) was that the dissociation constant for the complex was found to be far too weak to account for the lumazine protein effect on the bioluminescence as originating from preformed stable complexes with native luciferase. For example, for *P. phosphoreum* luciferase the dissociation constant is around 200 μM. A stronger complex was observed with *V. harveyi* luciferase where, in contrast, the bioluminescence interaction is weaker, and no complexation occurs at all with *V. fischeri* luciferase.

The required interaction, then, must be with one of the reaction intermediates (Lee, 1990). There are two intermediates that are metastable and amenable to study. The first is luciferase peroxyflavin (previously called "intermediate II"), formed by reaction of the $FMNH_2$ and O_2 on the luciferase in the absence of aldehyde (Hastings & Gibson, 1963). The other candidate is the "fluorescent transient", a highly fluorescent species formed in the course of the bioluminescent

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[1] Abbreviations: FMN, flavin mononucleotide; LumP, lumazine protein; ϕ, rotational correlation time; τ, fluorescence decay lifetime; Q_F, fluorescence yield; SD, standard deviation.

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reaction with aldehyde (Matheson et al., 1981; Matheson & Lee, 1983; Kurfuerst et al., 1984). This fluorescent transient is believed to be the emitter of the bioluminescence from the *V. harveyi* luciferase reaction, because its fluorescence spectrum is the same as the bioluminescence and the kinetics of the decay of the fluorescent transient is linked to the bioluminescence kinetics. Luciferase peroxyflavin and the fluorescent transient have the same absorption spectrum typical of a dihydroflavin electronic structure, with a maximum at 374 nm in the case of *V. harveyi* luciferase (Lee et al., 1989a).

Another fluorescent flavin–luciferase species is produced if the luciferase peroxyflavin is subject to photochemical irradiation in the 374-nm absorption band (Balny & Hastings, 1975). The irradiation causes the bioluminescence activity to be diminished, but the absorption spectrum is unchanged (Lee et al., 1988). This "luciferase photoflavin" is highly fluorescent, with a fluorescence maximum about 10 nm to longer wavelength than the bioluminescence, again as measured for *V. harveyi* (Lee et al., 1990a). The fluorescence dynamics properties of the *V. harveyi* luciferase photoflavin and the fluorescent transient are indistinguishable, both having an almost single exponential decay of fluorescence, τ = 10.2 ns (2 °C), and ϕ = 90 ns, but the photoflavin is much more stable.

These properties made the luciferase photoflavin an attractive subject for the first attempt at an energy-transfer investigation with lumazine protein (Lee et al., 1989a). This was by measuring the emission anisotropy decay in the mixture of the two proteins. For the photoflavin in the presence of lumazine protein, the apparent anisotropy correlation time, ϕ, was lowered to 25 ns, to be compared with the expected value mentioned above of 90 ns. Energy transfer is known to provide a channel of anisotropy loss in addition to rotational diffusion (Fayet & Wahl, 1969; Spencer & Weber, 1970). The energy-transfer rate recovered from these experiments was 2×10^7 s^{-1}. It was concluded that energy transfer would not be competitive with the decay rate (1×10^8 s^{-1}) from the fluorescent transient excited state, if this photoflavin case was representative of the bioluminescence process.

How the exergonicity of the bioluminescence oxidation is transformed into the excited-state energy of the emitter is a critical question in the mechanism of bacterial bioluminescence. Some have suggested that the luciferase-catalyzed reaction generates a primary excited species that is not fluorescent but can transfer its energy to a nearby fluorophore (Raushel & Baldwin, 1989; Lee et al., 1991a). Alternatively, it has been postulated that the fluorescent transient is directly generated in its excited state and that lumazine protein, along with other "secondary fluorophores", subsequently become excited by energy transfer from this primary species (Kurfuerst et al., 1984). A dipole–dipole type of energy transfer is not likely to be efficient because there is only a small overlap between the purported donor's fluorescence (maximum 490 nm) and the absorption of the acceptor, lumazine protein (maximum 417 nm), although this deficiency could well be compensated for by parallel orientation of the respective transition moments or by close proximity between the pair (Lee et al., 1990b). The rate of energy transfer also has to be well in excess of the sum of all loss processes from the primary excited state, to account for the bioluminescence spectral shift and quantum yield enhancement observed on addition of lumazine protein (Gast & Lee, 1978).

This present work extends the energy-transfer study to the more relevant luciferase intermediates. In addition, we also examine the effect of lumazine protein on the fluorescence dynamics properties using several different types of luciferases to make the flavin–luciferase intermediates. The results of anisotropy decay measurements, in particular, demonstrate that lumazine protein has a strong interaction with the *Photobacterium leiognathi* fluorescent transient and this parallels its effect on the bioluminescence with this luciferase.

Materials and Methods

The types of bioluminescent bacteria used were *V. harveyi* strain MAVA, an aldehyde-deficient dark mutant of strain MAV (O'Kane et al., 1986), *V. fischeri* strain "Pony" (Lee et al., 1990a), *P. phosphoreum* strain A13 (Gast & Lee, 1978), and *P. leiognathi* strain S1 (O'Kane et al., 1985). The luciferases were all purified to highest specific activity by use of HPLC methods (O'Kane et al., 1986). Lumazine protein was from *P. phosphoreum* strain A13 and was purified as described by O'Kane et al. (1985). Luciferase concentrations were assayed by absorbance assuming the ϵ(280 nm) = 85000 M^{-1} cm^{-1} for each type; for the lumazine protein, the visible absorbance maximum is 417 nm and ϵ(417 nm) = 10300 M^{-1} cm^{-1} (O'Kane & Lee, 1985). All chemicals were the best commercial grades.

The preparation of the luciferase peroxyflavin was by a small modification of the method described for *V. harveyi* (where it was called the "low-fluorescence intermediate II"; Lee et al., 1988). To the luciferase (0.5 mL, 400 μM) in standard buffer (50 mM P_i, pH = 7.0) containing 2-mercaptoethanol (10 mM) was rapidly added $FMNH_2$ (0.5 mL, 400 μM). Dodecanol (a stabilizing agent) was then added to bring its concentration to 200 μM. All procedures were carried out at 0–2 °C. The luciferase peroxyflavin was then separated from free FMN by using a Centrex centrifugal microfilter (Schleicher and Schell, Keene, NH) as previously described. The absorbance spectrum of the 0.5-mL luciferase peroxyflavin fraction was first measured, and then the fluorescent transient was prepared by the addition of 1 μL of tetradecanal saturated in ethanol. The absorbance spectrum was then rerun. These measurements were carried out below 5 °C. The amount of tetradecanal was determined by trial and error to be sufficient to convert all the peroxyflavin to fluorescent transient while still maintaining an optically clear solution.

Steady-State Spectra. Absorption spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer. Fluorescence and bioluminescence spectra were measured with an SLM 8000 spectrofluorometer with Glan-Thompson polarizers in the excitation and emission paths oriented in the magic-angle position (Lee et al., 1990a). All emission spectra were corrected for optical artifacts (e.g., self-absorption) and for the wavelength sensitivity of the instrument, by reference to the emission spectrum of quinine sulfate (Velapoldi & Mielenz, 1980). The quantum yield of fluorescence of quinine, 0.55, was also used as a reference standard (Parker, 1968).

Fluorescence Dynamics. Emission decay measurements were made with a laser system and single-photon counting electronics described in detail elsewhere (Lee et al., 1989b). The method of data collection and analysis was also as described elsewhere (Lee et al., 1988, 1989a,b, 1990a).

The procedure entails collecting three data sets of 512 channels, for the parallel, perpendicular, and prompt (laser pulse) signals. These three data sets together constitute a single "experiment" and were subject to the simultaneous analysis procedure. A preliminary analysis used the routine LIFETIME generously provided by Dr. Gary R. Holtom (Pacific North-West Laboratories, Richland, WA). This routine was useful in providing the "local fit", giving good starting values for the more complex "global" analytical methods (Knutson

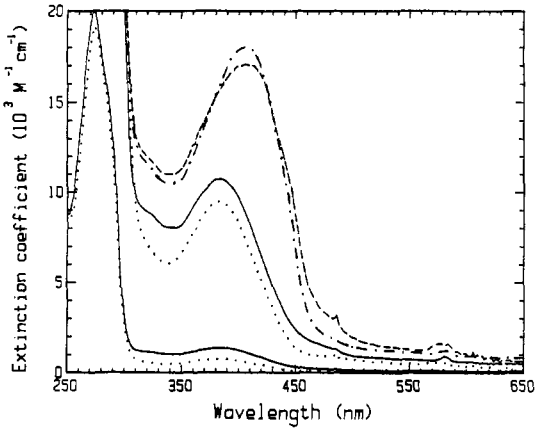

FIGURE 1: Absorption spectra for the luciferase fluorescent transients of *V. fischeri* (dots) and *P. leiognathi* (solid line). The dashed line is the observed spectrum for the mixture of *P. leiognathi* fluorescent transient and lumazine protein (both 55 μM), and the dot-dash spectrum is that calculated by addition of the spectra in Figures 1 and 2. The leftmost curves have been reduced 10×. All are at 2 °C, in 50 mM P_i, pH 7.0.

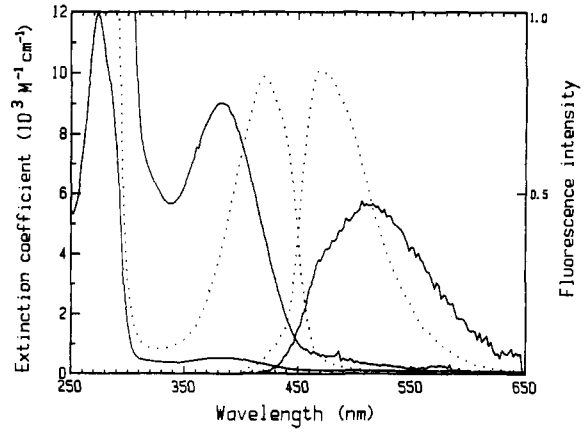

FIGURE 2: Absorption spectra (left curves) and fluorescence spectra (right curves) of the *V. fischeri* luciferase peroxyflavin (solid lines) and of *P. phosphoreum* lumazine protein (dotted lines). The leftmost curves are the absorption spectra reduced 10×.

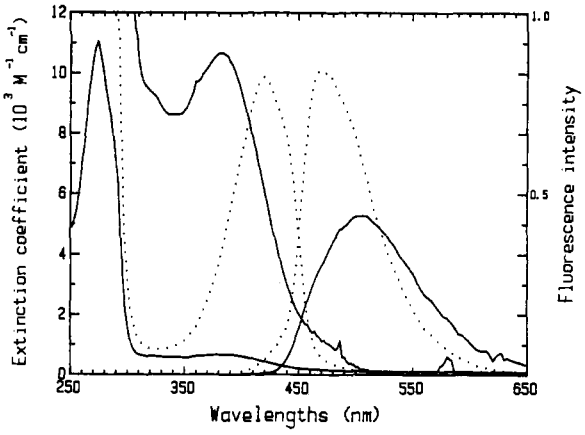

FIGURE 3: Absorption spectra (left curves) and fluorescence spectra (right curves) of the *P. leiognathi* luciferase peroxyflavin (solid lines) and of *P. phosphoreum* lumazine protein (dotted lines). The leftmost curves are the absorption spectra reduced 10×.

et al., 1983; Beechem et al., 1985) using the software package Globals Unlimited (Laboratory for Fluorescence Dynamics, University of Illinois, Urbana). The functions given in some figure legends are local fits to that particular data set. Only the relevant anisotropy parameters are given in the legends. The simultaneously obtained intensity parameters are close to those in the tables. All the results presented in the tables here are from the Globals program.

In the Globals routine, several experiments are analyzed by simultaneously holding, or "linking", certain parameters to the same values from one experiment to the next. As many parameters as possible are linked until the global (reduced) χ^2 becomes unacceptable (e.g., >1.4). For example, in Table II, the first two lines are for lumazine protein excited at 370 and 405 nm. The asterisks in the 405-nm line mean that the fluorescence lifetimes (τ's) and the initial anisotropy (r_0) are held the same (linked) for these two excitation wavelengths, but not the ϕ's. The global χ^2 is 1.17 and, for the two individual experiments, 1.08 and 1.30. For all parameters a 67% confidence interval, corresponding to one standard deviation (SD), was obtained according to the exhaustive search method (Beechem et al., 1991), and the result is the number in parentheses after that parameter, e.g., 25% for α_1 and 0.5 ns for τ_1, in the second line of Table II. It needs to be pointed out that the confidence interval is not usually symmetrical around the parameter estimate, and typical plots can be viewed elsewhere (Beechem & Gratton, 1990; Royer et al., 1990).

In fitting most of the data, sum of exponentials models are assumed (O'Connor & Phillips, 1984). The fluorescence intensity is

$$S(t) = \sum_i [\alpha_i \exp(-t/\tau_i)] \quad (1)$$

and the emission anisotropy is

$$r(t) = r_0 \sum_j [f_j \exp(-t/\phi_j)] \quad (2)$$

with $i = 3$ or 4 and $j = 1$ or 2; τ is the fluorescence lifetime, α the percent amplitude, ϕ_j is the rotational correlation time, and f_j the fractional intensity. The correlation times are assumed to be associated with all fluorescence decay times.

In those data sets where energy transfer is recognized (Table III), advantage is taken of the model analysis capability of the Globals package to add the energy transfer rate as an additional parameter.

RESULTS

Steady-State Spectra. The absorption spectra of the fluorescent transient products formed on reaction of tetradecanal with the luciferase peroxyflavins of *V. fischeri* and *P. leiognathi* are given in Figure 1. These spectra are measured in the time range 10–30 s following the addition of the tetradecanal, during which time the bioluminescence intensity is at a maximum. The bioluminescence signal is too weak to distort the absorption spectra, but the presence of the tetradecanal saturated in the aqueous buffer solution causes some scattering, which is the reason for the small slope in the base line. After allowing for this base-line distortion, it is concluded that the absorption spectra for these fluorescent transients are the same as for their luciferase peroxyflavins respectively (Figures 2 and 3), a conclusion already made for the *V. harveyi* fluorescent transient (Lee et al., 1989a). The absorption maxima for the *V. fischeri* and *P. leiognathi* species are at 5–8 nm longer wavelength than the 374-nm maximum of the *V. harveyi* fluorescent transient. The extinction coefficients in Figures 1–3 are determined from the absorption spectra after warming and recooling the samples to convert them to FMN and luciferase, with the assumption of 1:1 stoichiometry between the FMN and luciferase-bound flavin derivative.

The dashed line in Figure 1 is the spectrum for the *P. leiognathi* fluorescent transient after the addition of lumazine protein. The dash-dot line is the calculated addition spectrum by adding the lumazine protein absorption spectrum in Figure

Table I: Steady-State Spectral Parameters and Contribution to the Emission Signal at 460 nm[a]

	fluorescent transient			lumazine protein		
exn (nm)	extinction coeff (M^{-1} cm^{-1})	absorbance (cm^{-1})	460-nm signal (%)	extinction coeff (M^{-1} cm^{-1})	absorbance (cm^{-1})	460-nm signal (%)
		V. fischeri				
370	8500	0.17	28	2700	0.08	72
405	6800	0.14	9	8500	0.26	91
		P. leiognathi				
370	10400	0.31	31	2700	0.10	69
405	8600	0.26	13	8500	0.34	87
		V. harveyi				
370	10040	0.30	30	2700	0.10	70
405	7000	0.21	14	8500	0.31	86

[a] Conditions: 2 °C, pH 7.0, 50 mM P_i; emission 460 nm. Fluorescent transient concentration: *V. fischeri*, 20 μM; *P. leiognathi*, 30 μM; *V. harveyi*, 30 μM. The concentration of lumazine protein is 30, 36, and 36 μM, respectively. The percent signals are calculated for a mixture by assuming no change in steady-state parameters.

2 to the solid line in Figure 1. There is no significant difference between the observed and calculated spectra, which implies an absence of electronic ground-state interaction within the complex of fluorescent transient and lumazine protein.

For three of the types of luciferases used in this work, the fluorescent transients have half-lives of several hours at 2 °C in these buffer solutions containing dodecanol as a stabilizing agent. With *P. phosphoreum* luciferase a fluorescence transient product can be detected by its fluorescence dynamics properties (see later), but it is too unstable for its steady-state spectra to be accurately recovered.

Figures 2 and 3 present the absorption spectra of, respectively, the *V. fischeri* and *P. leiognathi* luciferase peroxyflavins and, after the addition of tetradecanal, the fluorescence spectra of their fluorescent transients. The peroxyflavins themselves are not fluorescent or only weakly so, mainly contributed to by a small amount of the free FMN breakdown product (Lee et al., 1988). After subtracting this FMN contribution, it is found that, for both of these luciferases, the fluorescence spectrum of their fluorescent transient and their bioluminescence spectrum are the same. Also plotted (dotted line) in Figures 2 and 3 for comparison are the absorption and fluorescence spectra of *P. phosphoreum* lumazine protein. The fluorescence quantum yield for lumazine protein is 0.58 and for the fluorescent transients 0.28 (*V. fischeri*) and 0.33 (*P. leiognathi*); the areas of the fluorescence spectra are scaled to correspond to these yields.

The fluorescence dynamics measurements are with the excitation at 370 or 405 nm and the emission at 460 nm. At 460 nm even with the widest slit widths used, 23 nm, interference from the fluorescence of the free FMN produced by the fluorescent transient's decay is practically eliminated. In a mixture of the lumazine protein and fluorescent transient, if no interaction occurs between the proteins that leads to a change in the emission properties of either of the fluorophores, then the relative contribution of each to the total 460-nm emission can be estimated from the data in Figures 2 and 3. For example, in Figure 2, at 460 nm a 20-nm slit width accepts 24% of the lumazine protein fluorescence spectrum and 9% of the *V. fischeri* fluorescent transient spectrum. At 405 nm, the two chromophores have about the same extinction coefficient, but at 370 nm the extinction of the fluorescent transient is 3.6 times that of lumazine protein.

Table I presents calculations for the intensity contributions for some typical experimental conditions, under the assumption above of no interactions. For example, in the first line, although the *V. fischeri* fluorescent transient has an initial

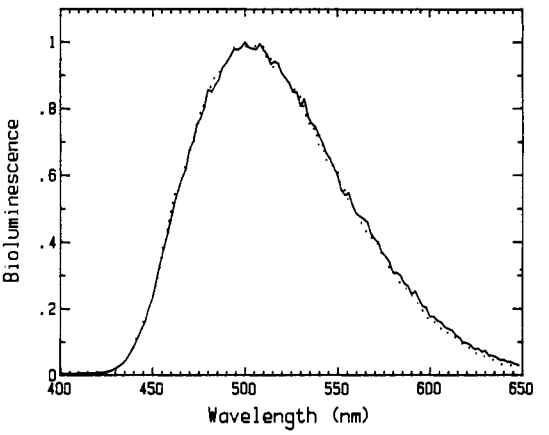

FIGURE 4: Bioluminescence spectrum (no excitation) from *V. fischeri* luciferase fluorescent transient (37 μM) without (solid line) or with 30 μM lumazine protein (dots); 2 °C, 50 mM P_i, pH 7.0.

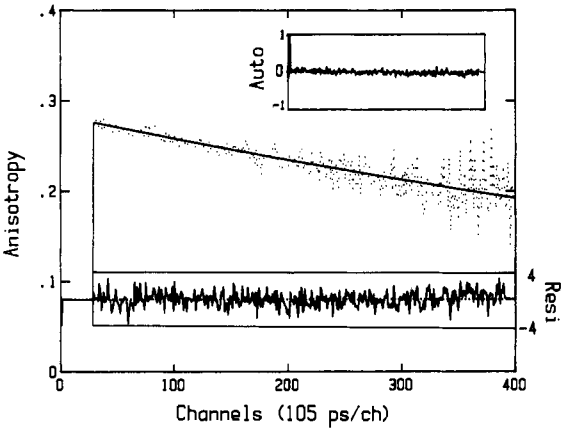

FIGURE 5: Fluorescence anisotropy decay of the *V. fischeri* fluorescent transient (37 μM). The data (dots) are fitted by the function represented by the line $r(t) = 0.36 \exp(-t/150)$; the time units are ns. The $\chi^2 = 1.24$; Auto = autocorrelation function; Resi = weighted residuals. Excitation 405 nm, emission 460 nm, 2 °C, 50 mM P_i, pH 7.0.

concentration of 30 μM, the calculation is made for an average concentration of 20 μM, considering that the transient decays during the measurement. Then in the mixture, the transient will contribute 28% of the total signal and lumazine protein 72%, for excitation 370 nm and emission 460 nm. The remaining lines are derived by the same procedure.

In the experiments a path length of 10 mm for excitation and 3 mm for emission was used. If the concentration of each component is typically 30 μM, this would lead to a total absorbance of 0.4 cm^{-1} at 405 nm and 0.33 cm^{-1} at 370 nm, giving rise to a nonlinearity with concentration or "geometrical" effect on the fluorescence signals. However, this would affect the two components equally, as would the smaller amount of self-absorption due to the lumazine protein absorbance of 0.1 cm^{-1} at 460 nm. Another artifact to take account of is depolarization due to self-absorption, but this could not be detected at the concentrations used for these studies.

Fluorescence Dynamics and Bioluminescence. V. fischeri. Figure 4 is the bioluminescence spectrum from the reaction of the *V. fischeri* luciferase peroxyflavin with tetradecanal, by itself (solid line) or with the addition of lumazine protein (dots). The two bioluminescence spectra are identical, lumazine protein at 30 μM has no effect on the spectrum from this strain "Pony" of *V. fischeri*, and both have a spectral maximum at 505 nm. The same result was found in an earlier study using the luciferase from *V. fischeri* strain 399, which

Table II: Fluorescence Dynamics Parameters for the *V. fischeri* Luciferase Intermediates Alone and in the Presence of Lumazine Protein[a]

	fluorescence							anisotropy			
exn (nm)	α_1 (%)	τ_1 (ns)	α_2 (%)	τ_2 (ns)	α_3 (%)	τ_3 (ns)	f_1	r_0	ϕ_1 (ns)	ϕ_2 (ns)	χ^2
					LumP (20 μM)						
											(global) 1.17
370	75 (20)	15.0	18 (7)	7.1	7 (C)	0.9		0.288	24.4 (1.0)		1.08
405	94 (25)	* (0.5)	2 (C)	* (1.0)	4 (C)	* (0.26)		* (0.006)	19.4 (1.2)		1.30
											(global) 1.36
370	70 (2)	14.5	2 (2)	3.0	28 (90)	0.1		0.329 (0.005)	23.2		1.39
405	70 (2)	* (0.17)	4 (2)	* (2.5)	26 (90)	* (0.2)		0.323 (0.005)	* (1.1)		1.38
					Luciferase (100 μM) + LumP (20 μM)						
											(global) 1.09
370	66 (31)	14.8	22 (11)	7.2	12 (6)	1.1		0.279 (0.009)	24.7		1.08
405	89	*	5	*	6	*		0.307 (0.014)	*		1.17
370[b]	* (42)	*	* (3)	*	* (4)	*		0.312 (0.012)	*		1.11
405[b]	63 (30)	* (0.3)	26 (9)	* (1.0)	11 (15)	* (0.75)		0.283 (0.020)	* (2.0)		1.07
					Luciferase Photoflavin (200 μM)[c]						
380	49	9.4	14	2.9	36	0.4		0.36	142		1.15
					Luciferase Fluorescent Transient (37 μM)						
											(global) 1.28
370	69 (24)	10.0 (0.1)	10 (2)	3.1	21 (20)	0.39		0.328 (0.004)	150		1.25
405	58 (C)	9.7	11 (2)	*	31 (9)	*		0.356	*		1.24
370	50 (C)	*	12 (3)	*	38 (10)	*		*	*		1.28
405	68 (C)	* (0.08)	6 (2)	* (0.8)	26 (8)	* (0.13)		* (0.0025)	* (15)		1.40
					Fluorescent Transient (30 μM) + LumP (30 μM)[d]						
											(global) 1.26
370	57 (18)	14.4	34 (14)	7.8	9 (3)	0.87		0.305 (0.005)	25.8		1.38
370	49 (C)	*	40 (C)	*	11 (3)	*		0.294 (0.005)	*		1.26
370	52 (C)	*	32 (C)	*	16 (4)	*		0.281 (0.0045)	*		1.21
405	81 (C)	* (0.3)	11 (14)	* (0.5)	8 (3)	* (0.32)		0.313 (0.0045)	* (0.7)		1.26
					Fluorescent Transient (30 μM) + LumP (30 μM)[e]						
											(global) 1.26
370	56 (C)	14.4	34 (C)	7.8	10 (C)	0.9	0.86 (0.04)	0.303 (0.006)	22.6	150	1.36
370	49 (C)	*	40 (C)	*	11 (C)	*	0.89 (0.03)	0.295 (0.005)	*	*	1.32
370	53 (34)	*	32 (C)	*	15 (8)	*	0.93 (0.04)	0.286 (0.005)	*	*	1.23
405	81 (31)	* (0.3)	10 (13)	* (0.8)	9 (5)	* (0.4)	0.95 (0.03)	0.320 (0.005)	* (F)	* (F)	1.17
					After Warming and Cooling						
370	62 (57)	14.0 (1.1)	24 (25)	5.9 (2.9)	14 (14)	0.6 (1.0)		0.268 (0.010)	22.9 (2.4)		1.15

[a] Conditions: 2 °C, 50 mM P_i, pH 7.0; emission = 460 nm; LumP = lumazine protein; * = linked parameter; F = fixed parameter; f_1 = fractional contribution from the amplitude of the ϕ_1 term. The numbers in parentheses are the 67% confidence intervals from the rigorous method of evaluation of errors; C = correlated, error estimation indeterminate. [b] With Corning 3-74 filter to eliminate stray scattered light. [c] From Lee et al. (1990a). [d,e] Same sample, same measurement, just with a different analysis.

has an emission maximum at 490 nm (Lee, 1982).

Figure 5 is the emission anisotropy decay of the *V. fischeri* fluorescent transient, and it is seen from the plots of the residuals and autocorrelation that the data can be accurately fitted with a monoexponential function (the smooth line) with a correlation time, ϕ, = 150 ns. In this and the following figures, the fit is taken only from the first or second channel after the maximum of the excitation pulse. In Figure 6 the fluorescent transient is mixed with lumazine protein and the overall decay becomes more complex. The fitted line is a biexponential anisotropy decay function with correlation times of 23 and 170 ns, values nearly the same as for the separate protein fluorophores.

Quantitative analysis of the *V. fischeri* experiments are in Table II. The top four lines are for lumazine protein samples from two different preparations, and the results show that the preparations do not differ significantly in their parameters. That is, the fluorescence decay is largely by a single-exponential lifetime averaging 14.7 ns (SD = 0.5), with one correlation time of average 22.6 ns (SD = 2.3) and initial anisotropy 0.31. A change of excitation wavelength in this range, 370–405 nm, does not change these parameters. It should be pointed out that while the rigorous method of error analysis yields satisfactory confidence intervals for most parameters, the fluorescence amplitudes (α) are often found to have confidence intervals in excess of 100% or even produce χ^2 that

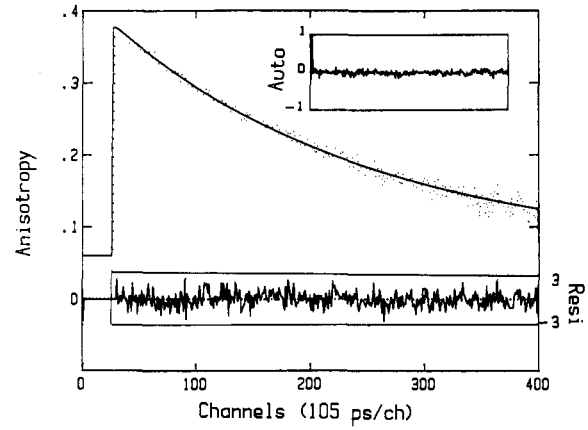

FIGURE 6: Fluorescence anisotropy decay of the *V. fischeri* fluorescent transient (37 μM) with lumazine protein (30 μM). The line is the function $r(t) = 0.34 \exp(-t/23) + 0.12 \exp(-t/170)$; $\chi^2 = 1.14$. Excitation 405 nm, emission 460 nm, 2 °C, 50 mM P_i, pH 7.0.

are flat because of high correlation. In such a case no error estimate can be made, and this is denoted by a "C" after that parameter. This problem of high correlation of the amplitude parameters is one generally encountered in fluorescence dynamics analysis (Grinvald & Steinberg, 1974).

The next set of four experiments, "luciferase (100 μM) + LumP (20 μM)", shows that no protein–protein complex is

formed between lumazine protein and *V. fischeri* luciferase in the native state, because association would yield an increase in the ϕ accompanied by a slight (1-ns) lowering of τ, if comparable to the results with *V. harveyi* luciferase (Lee et al., 1989b). There is no indication of scattered light distorting the data at early times, because insertion of the color filter did not change the parameters.

The next result for luciferase photoflavin is taken from the previous work in order to compare its parameters with the fluorescent transient (Lee et al., 1990a). As already found for *V. harveyi*, the *V. fischeri* photoflavin and fluorescent transient have very similar fluorescence parameters, a major τ = 9.7 ns (SD = 0.1 ns) and ϕ = 150 ns (SD = 15 ns) at 2 °C.

The four lines starting at α_1 = 69% are from measurements of this fluorescent transient made in chronological order, starting less than 30 min and extending to 3 h, following the reaction of the peroxyflavin with tetradecanal. The time taken for each experiment is typically about 40 min. In the 3-h time period the fluorescence signal (460 nm) has decayed by about half and FMN is formed, as reflected by the absorption spectrum. No change in the parameters is seen in this time period. The same chronology applies to all the experiments in Tables II and III.

The next set of experiments in Table II is for the mixture of fluorescent transient and lumazine protein. The initial concentration of the fluorescent transient from its absorbance is 30 μM, so its average concentration during the 3-h measuring time on the laser is approximately 20 μM. The analysis of this set of experiments is made under two assumptions. The first is that the fluorescence consists of three decay components and the anisotropy one component. From this result it could be concluded that the anisotropy decay reflects only lumazine protein (ϕ_1 = 25.8 ns). This is not so unexpected, since the lumazine protein fluorescence should contribute 72% of the total intensity (Table I).

Alternatively, the data can be analyzed by assuming two independent rotators with ϕ's fixed at the values for free lumazine protein and fluorescent transient, respectively. On statistical grounds the fit is equally good. These two decay models used illustrate the inherent difficulty of unrestricted analysis of fluorescence dynamics of mixtures. The anisotropy parameters are highly correlated, and a wide range of values, especially for ϕ_2, can give equally good fits.

The fractional contribution, c_i, by a component i to the steady-state fluorescence intensity is

$$c_i = \alpha_i\tau_i / \sum_i \alpha_i\tau_i \qquad (3)$$

For the ratio of the fractional contributions of lumazine protein (c_1) to fluorescent transient (c_2), the average result for the three lines starting at α_1 = 57% is 860/228 = 3.8, to be compared to the result found from the steady-state spectra, 72/28 ~ 2.6, in Table I. The latter result of course has considerable uncertainty because it depends on the estimate of the average fluorescent transient concentration being 20 μM. The fractional anisotropy of the lumazine protein f_1 shows a consistent behavior, increasing with time and with excitation from 370 to 405 nm. After warming and recooling, which converts all the fluorescent transient to FMN bound to the luciferase, the average ϕ = 22.9 ns drops to the value for lumazine protein itself.

Evidence for Interprotein Energy Transfer. Figure 7 shows the emission anisotropy decay for a mixture of lumazine protein with the fluorescent transient from *V. fischeri* (upper solid line), *V. harveyi* (dots), and *P. leiognathi* (lower solid line). A dramatically faster rate of anisotropy loss is seen for the *P. leiognathi* case than for either of the two *Vibrio* luciferases. For clarity, noisy data in channels before the position of the excitation pulse maximum, which is taken as t = 0, have been erased. Each curve starts at approximately the same value of anisotropy, the initial anisotropy, r_0 = 0.36. The *P. leiognathi* anisotropy shows at least two decay processes: the major proportion of decay events is fast, and a minor one is distinctly slower. A fast decay is also recovered in the analysis of the *V. harveyi* experiment, but it has a small amplitude and is hard to see in this figure. It is absent altogether in the *V. fischeri* case.

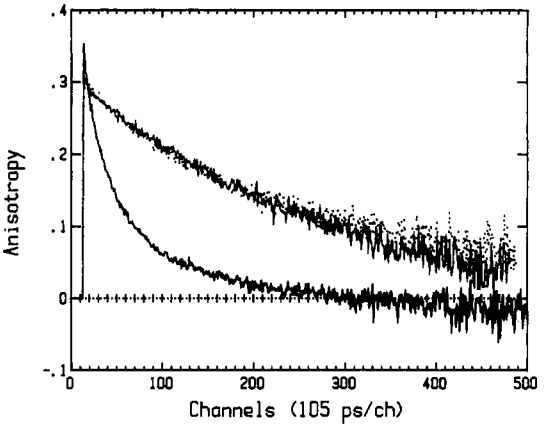

FIGURE 7: Fluorescence anisotropy decay for the fluorescence transients from the three types of luciferase in the presence of 30 μM lumazine protein. The luciferases used and initial concentrations are as follows: *V. fischeri*, 30 μM (top solid line); *V. harveyi*, 36 μM (dots); *P. leiognathi*, 55 μM (lower solid line). Excitation 370 nm, emission 460 nm, 2 °C, 50 mM P_i, pH 7.0.

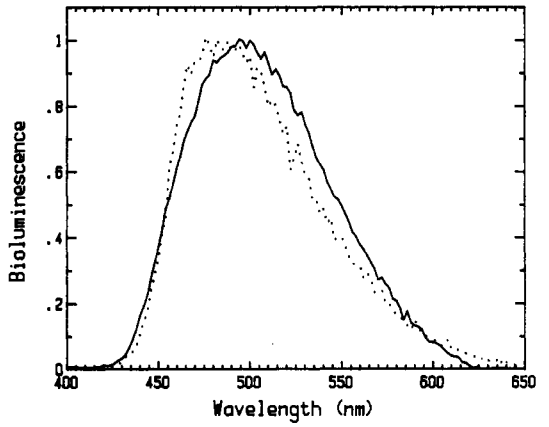

FIGURE 8: Bioluminescence spectrum (no excitation) from *P. leiognathi* luciferase fluorescent transient (40 μM) without (solid line) or with 55 μM lumazine protein (dots). 2 °C, 50 mM P_i, pH 7.0.

The interpretation is that a large proportion of the *P. leiognathi* fluorescent transient forms a protein–protein complex with the lumazine protein in which energy transfer from the initially excited donor, the flavin on the luciferase, is efficiently deposited onto the associated lumazine ligand. In *V. harveyi*, the proportion of fluorescent transient complexing is much smaller but also gives rise to the faster decay rate. The slower decay is from the *V. harveyi* luciferase–lumazine protein complex (K_d ~ 40 μM). For *V. fischeri*, the decay resolves into that expected for the two independent rotors (e.g., Figure 6).

P. leiognathi. The case of the *P. leiognathi* fluorescent transient is of course the most revealing, and this will be examined in detail. Figure 8 is the bioluminescence spectrum

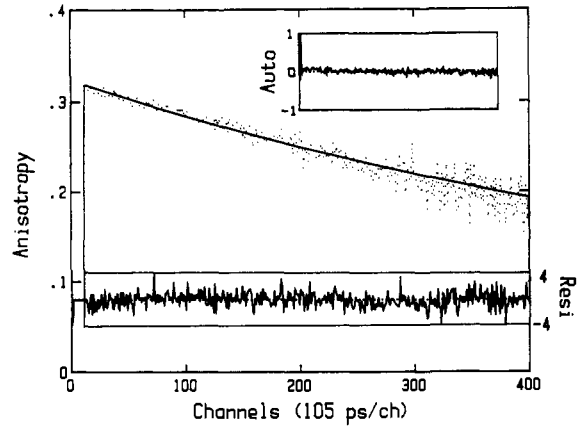

FIGURE 9: Fluorescence anisotropy decay of the *P. leiognathi* fluorescent transient (40 μM). The line is the function $r(t) = 0.31 \exp(-t/79)$; $\chi^2 = 1.06$. Excitation 405 nm, emission 460 nm, 2 °C, 50 mM P_i, pH 7.0.

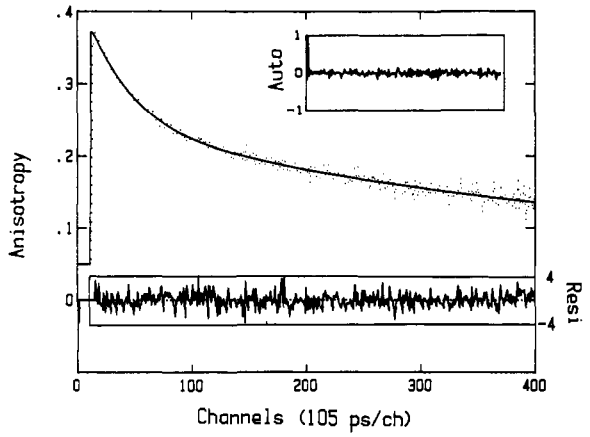

FIGURE 10: Fluorescence anisotropy decay of the *P. leiognathi* fluorescent transient (40 μM) with lumazine protein (55 μM). The line is the function $r(t) = 0.1 \exp(-t/3.1) + 0.22 \exp(-t/40)$; $\chi^2 = 1.25$. Excitation 405 nm, emission 460 nm, 2 °C, 50 mM P_i, pH 7.0.

generated by addition of tetradecanal to luciferase peroxyflavin alone (solid curve), or in the presence of lumazine protein (dots). Lumazine protein shifts the bioluminescence maximum about 18 nm to shorter wavelength, similar to the effect reported before for the bioluminescence from a different strain (477) of *P. leiognathi* luciferase (Lee, 1982). Figure 9 shows the anisotropy decay of the *P. leiognathi* luciferase fluorescent transient, and Figure 10 is with lumazine protein. Here the excitation wavelength is 405 nm, so most of the absorption is into the lumazine chromophore, and so the amplitude of the rapidly decaying anisotropy due to energy transfer from the flavin is smaller than in Figure 6, where the excitation at 370 nm is mostly into the bound flavin chromophore.

Table III collects the fluorescence dynamics parameters for the *P. leiognathi* experiments. The first set of four experiments is for a mixture of the native luciferase with lumazine protein. The anisotropy decay contains two components, and since one of these is the free lumazine protein, ϕ_1 can be fixed at 22.6 ns for the analysis. As the lumazine protein concentration is increased, the fractional contribution f_1 also increases. No significant effect of incubation time can be seen. By use of the same method as described before for *V. harveyi* (Lee et al., 1989b), a dissociation constant for the luciferase–lumazine protein complexation is recovered and is about 15 μM. The value $\phi_2 = 65$ ns (SD = 37) is consistent with the rotation of the 100-kDa protein–protein complex.

As with the *Vibrio* luciferases, the *P. leiognathi* photoflavin and fluorescent transient have similar properties, viz., a dominant $\tau_1 \sim 10$ ns with minor contributions from shorter lifetimes and a monoexponential anisotropy decay. The *P. leiognathi* fluorescent transient $\tau_1 = 10.6$ ns (SD = 0.16) is 1 ns longer than the τ_1 of the *V. fischeri* fluorescent transient, but what is of particular note is that the *P. leiognathi* $\phi = 80$ ns, is close to that expected for the rotational diffusion of luciferase having a mass of 77 kDa. This is in contrast to the ϕ for the *Vibrio* photoflavins and fluorescent transients, particularly so for *V. fischeri* (150 ns). The *Vibrio* intermediates have been shown to form dimers (150 kDa) whereas the *P. leiognathi* ones remain monomeric (Lee et al., 1991b).

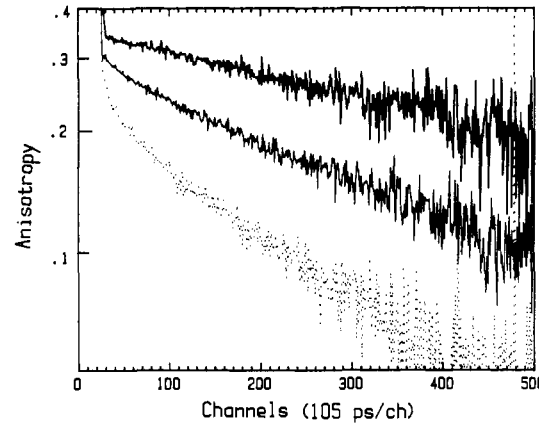

FIGURE 11: Fluorescence anisotropy decay (log scale) for *P. leiognathi* luciferase species. Luciferase photoflavin (top solid line) with excitation 405 nm (excitation at 370 nm gives the same result); luciferase peroxyflavin (36 μM) with lumazine protein (36 μM) with excitation at 405 nm (lower solid line) and 370 nm (dots). Emission 460 nm, 2 °C, 50 mM P_i, pH 7.

For the mixture of *P. leiognathi* luciferase peroxyflavin and lumazine protein, two correlation times are observed (Figure 11). The anisotropy decays are plotted in Figure 11. For comparison, the anisotropy decay of the *P. leiognathi* photoflavin is shown here because the peroxyflavin itself is only weakly fluorescent. Analysis of the data shows the presence of two ϕ's: the shorter $\phi_2 = 5.1$ ns is attributed to anisotropy loss due to energy transfer from the peroxyflavin to the lumazine within the luciferase–lumazine protein complex, and the longer $\phi_1 = 47$ ns (SD = 12) is consistent with the $\phi_2 = 65$ ns for the native luciferase–lumazine protein complex. The results also indicate that the lumazine protein is associated with the peroxyflavin to about the same extent as with the native luciferase. The fractional contribution of the energy-transfer component increases with time into the experiment. When the excitation is at 405 nm mainly into the lumazine, the contribution from energy transfer (f_2) becomes much smaller.

For the mixture with the fluorescent transient of *P. leiognathi* the longer correlation time around 40 ns is not seen at all for 370-nm excitation but is still there for 405 nm. The anisotropy decay is more complex than before, and we take advantage of the model analysis facility in the Globals Unlimited software to insert an additional rate parameter, k_T, to account for the rate of energy transfer between the donor and acceptor:

$$\text{FT*·LumP} \rightarrow \text{FT·LumP*} \qquad \text{rate} = k_T \qquad (4)$$

Two sets of experiments are shown in Table III, one with an initial concentration of the fluorescent transient at 55 μM with the same concentration of lumazine protein, and the second with both at 44 μM. For the 55 μM case and excitation at 370 nm there are two fast decays of anisotropy, $\phi_1 = 10$–16 ns and $\phi_2 = 3.1$ ns. At 405-nm excitation, ϕ_1 lengthens to

Table III: Fluorescence Dynamics Parameters of the *P. leiognathi* Intermediates Alone and in the Presence of Lumazine Protein[a]

	fluorescence						anisotropy				
exn (nm)	α_1 (%)	τ_1 (ns)	α_2 (%)	τ_2 (ns)	α_3 (%)	τ_3 (ns)	r_0	f_1	ϕ_1 (ns)	ϕ_2 (ns)	χ^2
Luciferase (20 μM) + LumP (20 μM)											
											(global) 1.192
375[b]	61 (C)	14.3	20 (20)	6.4	19 (11)	0.62	0.276 (0.007)	0.39 (0.46)	22.6	65	1.13
375[c]	69 (C)	*	18 (21)	*	13 (7)	*	0.266 (0.006)	0.58	*	*	1.16
375[d]	75	*	15	*	10	*	0.278	*	*	*	1.14
375[e]	* (15)	* (0.33)	* (5)	* (1.5)	* (10)	* (0.3)	* (0.004)	* (0.18)	* (F)	* (37)	1.23
Luciferase Photoflavin (28 μM)											
											(global) 1.29
370	42 (7)	10.3	15 (C)	2.4	43 (C)	0.3	0.345		85.4		1.32
405	51 (C)	*	7 (C)	*	42 (C)	*	* (0.004)		*		1.29
370	26 (4)	*	17 (C)	*	57 (C)	*	0.330 (0.005)		*		1.27
405	48 (C)	* (0.09)	12 (C)	* (0.35)	40 (C)	* (0.1)	0.339 (0.006)		* (6.0)		1.34
Luciferase Fluorescent Transient (40 μM)											
											(global) 1.205
370	71	10.6	8	3.4	21	0.58	0.277 (0.003)		79.2		1.21
370	* (38)	*	* (3)	*	* (6)	*	0.317		*		1.18
370	63 (33)	*	10 (5)	*	27 (9)	*	*		*		1.25
405	88 (47)	* (0.16)	3 (2)	* (2.6)	11 (5)	* (0.36)	* (0.002)		* (3.4)		1.25
Luciferase Peroxyflavin (36 μM) + LumP (36 μM)											
											(global) 1.23
370	58 (55)	13.8	18 (40)	8.0	24 (19)	0.6	0.293 (0.043)	0.70 (0.18)	47	5.1	1.20
370	69 (60)	*	12 (42)	*	19 (18)	*	0.314 (0.022)	0.41 (0.08)	*	*	1.28
370	74 (49)	*	11 (44)	*	15 (39)	*	0.303 (0.031)	0.31 (0.08)	*	*	1.35
405	83 (48)	* (0.9)	9 (40)	* (4.0)	8 (1)	* (0.4)	0.302 (0.019)	0.91 (0.15)	* (12)	* (1.4)	1.18
Luciferase Fluorescent Transient (55 μM) + LumP (55 μM)[f]											
											(global) 1.33
370	21 (8)	13.82	28 (44)	6.5	51 (79)	1.5	0.343	0.37	15.8 (9.8)	3.1	1.48
370	15 (14)	*	24 (30)	*	61 (68)	*	*	*	10.8	*	1.37
370	13 (C)	*	23 (C)	*	64 (C)	*	*	*	*	*	1.32
370	14 (14)	* (0.10)	23 (11)	*	63 (60)	*	* (0.015)	* (0.14)	* (5.8)	*	1.44
405	30 (3)	14.10 (0.07)	32 (21)	*	38 (63)	*	0.317	0.59	37	*	1.33
405	18 (9)	*	25 (27)	* (C)	57 (C)	* (0.4)	* (0.023)	* (0.07)	* (14)	* (0.7)	1.27
After Warming and Recooling											
											(global) 1.10
370	59 (38)	14.6	23 (22)	7.1	18 (7)	1.2	0.279	0.78 (0.12)	32	3.2	1.18
405	74 (49)	* (0.7)	11 (19)	* (2.9)	15 (5)	* (0.8)	* (0.070)	0.95 (0.12)	* (20)	* (2.6)	1.05
Luciferase Fluorescent Transient (44 μM) + LumP (55 μM)[f]											
											(global) 1.25
370	39 (13)	13.60 (0.14)	38 (18)	3.0	23 (C)	1.2	0.320 (0.033)	0.52 (0.18)	14.3 (4.7)	2.6	1.40
370	29	13.78 (0.12)	31	*	40	*	0.329 (0.033)	0.48 (0.22)	12.0 (7.5)	*	1.21
370	*	13.87 (0.10)	*	*	*	*	0.344 (0.032)	0.45 (0.20)	*	*	1.21
370	* (9)	*	* (33)	*	* (C)	*	0.342 (0.032)	*	*	*	1.30
405	38 (12)	13.98 (0.16)	35 (24)	* (6.9)	27 (C)	* (1.4)	0.270 (0.087)	0.69 (0.13)	24.0 (20)	* (1.3)	1.26
After Warming and Recooling											
											(global) 1.18
370	56 (27)	14.45	22 (26)	6.7	22 (15)	0.74	0.322	0.72 (0.13)	41	4.1	1.20
405	77 (37)	* (0.55)	11 (10)	* (2.5)	12 (10)	* (0.5)	* (0.13)	0.89 (0.09)	* (37)	* (3.0)	1.19
Mixture [Luciferase + FMN + LumP (44 μM)]											
											(global) 1.29
370	66 (49)	14.4	22 (23)	6.8	12 (7)	0.88	0.272 (0.037)		27.1		1.47
405	86 (54)	* (0.6)	6 (10)	* (2.3)	8 (8)	* (0.85)	0.315 (0.053)		* (8.5)		1.14
											(global) 1.20
370	67 (51)	14.5	22 (18)	6.9	11 (16)	1.0	0.319	0.83 (0.30)	27.2	0.4	1.29
405	86 (20)	* (0.7)	6 (12)	* (2.5)	8 (8)	* (1.0)	* (0.11)	0.99 (0.25)	* (9.0)	* (0.7)	1.14

[a] 2 °C, pH 7.0, 50 mM P_i, emission 460 nm. [b-d] The concentrations of lumazine protein are [b] 5 μM, [c] 10 μM, and [d] 20 μM. [e] After 2 h. [f] With an additional energy-transfer parameter (see text). C = correlated; F = fixed.

about 37 ns, corresponding again to the rotation of the protein–protein complex. The faster time (ϕ_2 = 3.1–2.6 ns) is not changed significantly by concentration of the species or excitation wavelength. For both sets of data the energy-transfer rate recovered is k_T = 1 ns^{-1} (SD = 1). Clearly, more experiments will be required to target this parameter more precisely. Even after the sample is warmed and recooled to destroy most of the fluorescent transient, the ϕ_2 fast decay is still present but only to a small extent.

Two analyses are shown of the mixture of native luciferase, lumazine protein, and FMN; the first model assumes no energy transfer and the second that energy transfer occurs reflected by ϕ_2, from the bound FMN to the lumazine. The result gives no support to this latter assumption.

V. harveyi. The top panel of Figure 12 shows that lumazine protein shifts the *V. harveyi* bioluminescence spectrum. Again as previously observed for a different strain of *V. harveyi*, the shift is not as much as is found with the *Photobacterium* luciferases but more than with *V. fischeri*. In the lower panel is the anisotropy decay of the fluorescent transient from *V. harveyi* luciferase, with (two lower curves) and without (top curve) lumazine protein. The picture is qualitatively the same

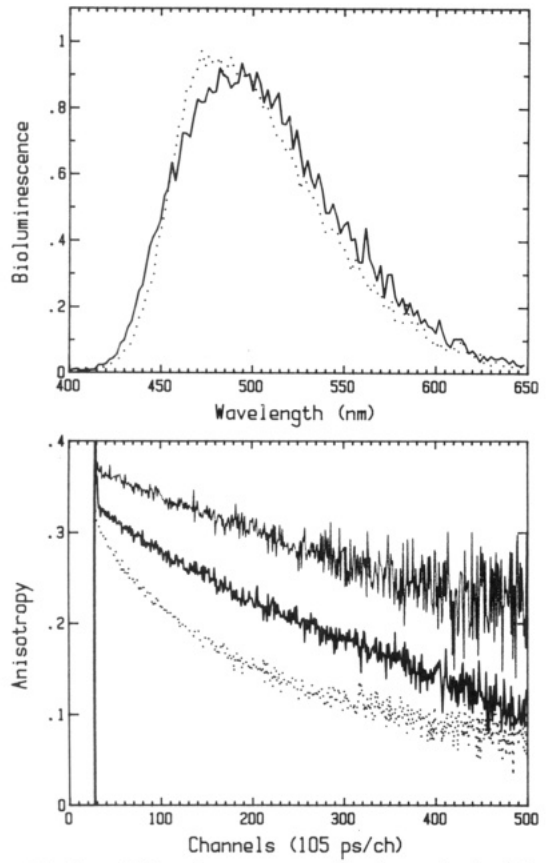

FIGURE 12: Top: Bioluminescence spectrum (no excitation) from *V. harveyi* luciferase fluorescent transient (50 μM) without (solid line) or with 50 μM lumazine protein (dots). Bottom: Fluorescence anisotropy decay of the *V. harveyi* fluorescent transient (50 μM) without (top solid line) or with 50 μM lumazine protein (lower solid line, excitation 405 nm; dots, excitation 370 nm). Emission 460 nm, 2 °C, 50 mM P_i, pH 7.0.

as with *P. leiognathi*, that is, one short and one longer correlation time in the presence of lumazine protein, with the relative amplitude, f_2, of the shorter correlation time much less than in the case of *P. leiognathi* (Figure 7).

A study of the *V. harveyi* intermediates alone and mixed with lumazine protein has been made and some results have already been published (Lee et al., 1990b). For brevity the results will not be presented in detail because the same energy-transfer parameters are seen as in Table III but less prominently. The anisotropy decay is complex, requiring a two-component fit. A fast correlation time around 2–6 ns is found but is of small amplitude, 0.02. A longer time, 40–60 ns, reflects the rotation of a protein–protein complex.

P. phosphoreum. The intermediates from *P. phosphoreum* luciferase are not able to be stabilized for longer than about 20 min. Therefore, it was not possible to obtain detailed fluorescence dynamics parameters. The bioluminescence shift in the presence of lumazine protein was almost the same as for the *P. leiognathi* reaction, as has been reported before (Lee, 1982). Immediate measurement of the fluorescence parameters following tetradecanal addition to a preparation of the luciferase peroxyflavin resulted in a recovery of a fluorescence lifetime of 9.6 ns and $\phi \sim 100$ ns. Attempts to recover data of sufficient quality from mixing with lumazine protein were not successful.

Discussion

Figure 13 pictures the complex of the *P. leiognathi* luciferase intermediate and lumazine protein in a specific association to enable rapid electronic energy transfer ($k_T = 1$ ns^{-1}) from the excited flavin to the lumazine. This accounts for the fast channel (ϕ_2) of anisotropy loss.

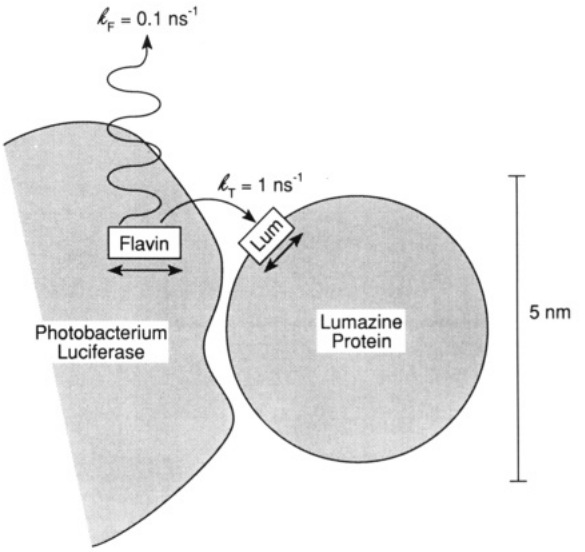

FIGURE 13: In the specific complex formed between the *Photobacterium* luciferase bioluminescence intermediate and lumazine protein, the ligands are placed so that rapid (1-ns^{-1}) energy transfer from the flavin donor to the lumazine acceptor outcompetes the fluorescence rate (0.1 ns^{-1}) from the flavin. The arrows represent arbitrary transition moment directions.

No association is evident in the case of the fluorescent transient of *V. fischeri* and lumazine protein because no fast anisotropy loss is observed. The decay of anisotropy in this mixture can be accounted for by the sum of the anisotropy decay functions of the two separate fluorophores. At the concentrations used of less than 50 μM, the average separation between the donor and acceptor in homogeneous solution is more than an order of magnitude greater than the Foerster distance (eq 7). Therefore, radiationless energy transfer could not take place without specific association of the two proteins.

In the case of the *V. harveyi* fluorescent transient and lumazine protein, a fast decaying anisotropy with a $\phi = 2$–8 ns is observed, and it is in the same range as for *P. leiognathi*. Its amplitude, f_2, however, is much smaller than for *P. leiognathi*. It is concluded that the protein–protein association is weaker in the *V. harveyi* case than in *P. leiognathi*, and this accounts for the lesser amount of bioluminescence spectral shift in the *V. harveyi* luciferase in vitro reaction with added lumazine protein. In the previous study with the photoflavin (Lee et al., 1989a), this fast correlation time was not observed either because no complexes were formed or because the energy transfer in these complexes was in fact slow.

These results show that energy transfer in a protein–protein complex of the luciferase intermediate and lumazine protein is a feasible mechanism by which the lumazine becomes excited in the bioluminescence reaction. No association or energy transfer is seen for *V. fischeri*, and there is no bioluminescence spectral shift with lumazine protein. Some effect is found with *V. harveyi* accompanied by a small amount of associated species, and a full effect and complexation is found for *P. leiognathi*. Whether the association specifically occurs only after the addition of aldehyde to form the fluorescent transient remains a question for more systematic measurement. For *P. leiognathi* native luciferase, the observed dissociation constant of 15 μM fully accounts for the lumazine protein bioluminescence interaction. However, the lumazine protein is just as effective at the 10's of micromolar concentration level with the *P. phosphoreum* bioluminescence, but the complexation with the native luciferase, as measured by changes in

ϕ, is very weak ($K_d \sim 200$ μM; Lee et al., 1989b).

The well-known Foerster mechanism of energy transfer by the weakly coupled interaction between the singlet–singlet electronic dipolar transitions of a donor and acceptor adequately accounts for the excitation transfer. The rate of transfer of energy by this mechanism is given by (Foerster, 1965)

$$k_T = k_F(R_0/R)^6 \quad (5)$$

where k_F is the fluorescence decay rate of the initially excited state, the donor, that transfers its energy to an acceptor, situated at a distance R from the donor. R_0 is called the critical Foerster distance at which

$$k_T = k_F \quad (6)$$

and is given by

$$(R_0)^6 = (2.8 \times 10^{-25})Q_F K^2[J\ (M^{-1}\ cm^3)] \quad (7)$$

where the numerical value is a product of a number of constants, including the refractive index of the solvent, in this case, water (1.33). Q_F, = 0.33, is the fluorescence quantum yield of the (flavin) donor in the absence of the (lumazine) acceptor, K^2 is an orientation factor, dependent on the angle between the dipole moment of the donor's emission transition dipole and the acceptor's absorption transition dipole, and J is the overlap integral of the fluorescence spectrum of the donor normalized to unit area, with the molar decadic extinction spectrum of the acceptor. This is evaluated in Figure 3 as $J = 2.0 \times 10^{-15}$ M^{-1} cm^3.

The first obstacle to the enumeration of R_0 is the knowledge of the orientation factor, K^2 (Dale, 1978). When the donor and acceptor are small molecules undergoing random encounters in homogeneous solution, their orientations can be assumed to be dynamically averaged in the times corresponding to the inverse rates k_F and k_T. The K^2 factor can then be readily evaluated as $K^2 = 2/3$. In the literature of the energy-transfer field even within proteins, this value is almost always assumed, and substituting in eq 7, $R_0(2/3) = 22$ Å. For the protein–protein case under present consideration, dynamic averaging probably cannot occur for the following reasons.

The anisotropy decays of both fluorophores under consideration, the ligands in lumazine protein and the *P. leiognathi* fluorescent transient when they are in their separate states, can be accurately fitted by a single-exponential process (Figure 9). The Stokes–Einstein equation for a spherical rotator is

$$\phi = M_r\eta(\bar{v} + h)/(RT) \quad (8)$$

where M_r is the molecular weight, η the solution viscosity, $\bar{v}$ the partial specific volume, h the protein's hydration (0.45 cm^3/g), R the universal gas constant, and T the absolute temperature. The correlation times for these two fluorophores correspond respectively to the rotational diffusion of the macromolecules as a whole (eq 8), 22 ns (Table II) and 80 ns (Table III), and the interpretation of this is that the bound ligands have no motion independent of the whole macromolecule, i.e., the lumazine and the flavin molecules are rigidly attached to their apoproteins.

In the complex of lumazine protein and the fluorescent transient of *P. leiognathi* luciferase, if the two proteins dock together tightly and specifically, then the flavin and the lumazine (the donor and acceptor) would be fixed in both separation and orientation (Figure 13). Energy transfer in such a fixed system is one of "restricted geometry" (Klafter & Drake, 1989), and such cases are of much current interest. Unfortunately, this circumstance does not allow a separation of the K^2 term from R_0, unless the three-dimensional structure of the complex was known. Only one macromolecular system has been examined in this regard. This was a model study where two hemes in hemoglobin were replaced by chlorophyllides and the separation of the energy-transfer pair was known accurately from the X-ray structure (Moog et al., 1984). The rate of energy transfer was found to be consistent with the accurately known separation and also allowed limits on the orientation of the pair to be estimated.

Some theoretical treatment of this case of donor and acceptor at a fixed distance and/or orientation is available (Dale, 1978; Tanaka & Mataga, 1979, 1982) and was recently extended and applied to intraprotein energy transfer in flavodoxin (Leenders et al., 1989). These more sophisticated approaches are not justified for these present studies until certain technical difficulties can be dealt with. For example, both absorption and emission transitions are mixtures of only approximately known amounts of the donor.

If we accept the result from Table III that $k_T = 1$ ns^{-1}, then an estimate of the separation of the donor and acceptor can be made. It is evident that the transition moments are not parallel because anisotropy is lost on energy transfer and there is no significant residual anisotropy. If the orientations were closer to orthogonal, the separation would need to be small to account for the energy-transfer rate. A value of $K^2 = 0.1$ would then lead to a minimum estimate of $R = 11$ Å. It needs to be noted that R is not substantially affected by rather wide choices of either K^2 or k_T, because of the sixth power dependence. If the separation was below 10 Å, then exchange and excitonic interactions would come into play, providing much faster channels of energy transfer (picoseconds). But no such interactions are evident in the absorption spectra of the mixture because the spectral sum is linear (Figure 1).

Another assumption made for the calculation is that in the protein–protein complex the Q_F of the donor (if the acceptor were absent) is unchanged from the uncomplexed state. From the Birks–Dyson approximation:

$$\epsilon = 10^{-4}Q_F/\tau \quad (9)$$

it is reasonable that the Q_F is not changed since the extinctions are additive in Figure 1.

Lumazine protein inclusion in the bioluminescence reaction of *P. leiognathi* luciferase not only changes the bioluminescence spectrum to that of the fluorescence of the bound lumazine but also increases the bioluminescence quantum yield about 2 times (O'Kane & Lee, 1986). For the mixture of fluorescent transient with lumazine protein, excitation at 370 nm in the flavin absorption band results in an increase of Q_F to about 0.45, to be compared with a value of 0.33 for the fluorescent transient alone. Technical difficulties, however, limit the accuracy of fluorescence quantum yield measurements in such mixtures; the absorbances are very high, for example, around 0.5, requiring many corrections. For the fluorescent transient

$$Q_F = k_R/(k_R + k_{NR}) \quad (10)$$

where k_R is the radiative rate constant and k_{NR} the nonradiative rate constants. The denominator is simply the inverse of the fluorescence lifetime, which is observed to have a weighted average value about 10 ns. Then the sum of the loss rates from the excited state of the fluorescent transient is 10^8 s^{-1}, and so this loss would not be competitive with $k_T = 10^9$ s^{-1}. The Q_F for lumazine protein separately is 0.58, so this fast energy-transfer rate would be predicted to enhance the bioluminescence quantum yield by 1.7, comparable to the value

2.4 reported for this case (O'Kane & Lee, 1986).

In this work we have shown that energy transfer occurs between the luciferase bioluminescence intermediates and lumazine protein in those instances where lumazine protein is most effective in the bioluminescence reaction, viz., *P. leiognathi* but not *V. fischeri*. This energy transfer reflects the presence of protein–protein complexes. The estimate of the energy-transfer rate, although preliminary, favors the suggestion that the bioluminescence excitation of lumazine protein is by a "photophysical" mechanism.

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