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Lumazine protein and the excitation mechanism in bacterial bioluminescence

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Abstract

The spectral properties of lumazine protein and mixtures with the intermediates of the bacterial luciferase reaction, are reviewed. Measurements of fluorescence dynamics in particular have been employed with the aim of elucidating the mechanism by which lumazine protein functions in the bioluminescence of the bacteria of the type *Photobacterium*. The reaction of bacterial luciferase with its substrates produces bioluminescence emission with a spectral maximum at 496 nm. This spectrum is the same as the fluorescence of a luciferase flavin intermediate in the reaction, called the Fluorescent Transient. When lumazine protein is also present in the reaction, however, the bioluminescence emission now corresponds to the fluorescence of lumazine protein, which has a maximum at 475 nm. From measurements of the decay of fluorescence anisotropy of lumazine protein alone and in mixtures with the luciferase fluorescent transient, it is shown that a protein–protein complex is formed and that there is rapid energy transfer between the flavin on the luciferase and the lumazine derivative bound to its protein. An approximate calculation estimates the rate of this energy transfer to be faster than $10^9\ s^{-1}$, and this would account for the efficient transfer of excitation from the flavin on the associated luciferase in the mixed protein bioluminescence reaction.

Keywords: Photobacterium; Bacterial luciferase; Flavin; Energy transfer; Emission anisotropy decay; Bioluminescence

1. Introduction

Molecular oxygen adds to $FMNH_2$ on bacterial luciferase to form a metastable product, luciferase peroxyflavin (4a-peroxy-1,5-dihydroFMN; FMNH-4a-OOH, Fig. 1) [1]. This compound has negligible fluorescence [2] but addition of an aldehyde, such as tetradecanal, produces a highly fluorescent intermediate, called the Fluorescent Transient, accompanied by bioluminescence emission [3]. Since the spectral distribution of the bioluminescence and the fluorescence of the fluorescent transient correspond, then it is concluded that this fluorescent state is the origin of the bioluminescence emission. Chemical and spectroscopic evidence have led to the suggestion that the fluorescent transient is luciferase-bound 4a-hydroxy-1,5-dihydroFMN (a flavin pseudo-base; FMNH-4a-OH, Fig. 1) [4].

When lumazine protein is also included in the bioluminescence reaction, the bioluminescence spectrum no longer corresponds to the fluorescence of the fluorescent transient but is shifted towards shorter wavelengths and can become the same as the fluorescence of the lumazine protein

Abbreviations: FMN, flavin mononucleotide; LumP, lumazine protein; ANS, 8-amino-1-naphthalenesulfonate; FWHM, full width at half maximum.

[5]. The amount of spectral shift depends on reaction conditions but even at 10 μM concentration of the added lumazine protein (21 kD), with the luciferase (76 kD) at about 1 μM, the bioluminescence can correspond to the fluorescence of the added lumazine protein. The fluorescence lifetime of the fluorescent transient is only 10 ns [6], so for the lumazine protein to have such an effect as a "sensitizer" at the 10 μM level, the lumazine derivative must have a proximity to the luciferase reaction site such as would occur if the two proteins formed a stable associated complex.

This paper reviews the spectroscopic properties of lumazine protein and mixtures with the luciferase intermediates. The aim of these studies has been to understand the mechanism of the bioluminescence excitation.

2. Spectral properties of lumazine protein

The absorption and fluorescence spectra of lumazine protein are shown in Fig. 2 [7]. The bound lumazine derivative (6,7-dimethyl-8-ribityl-

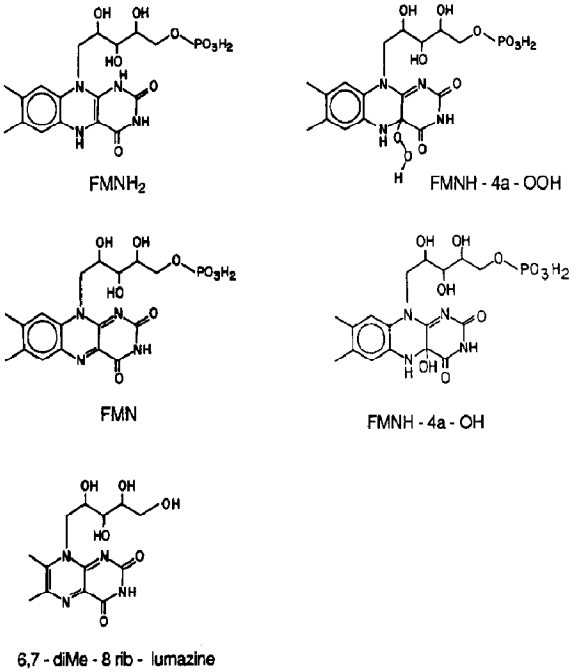

Fig. 1. Flavin and lumazine derivatives involved in the bacterial luciferase reaction.

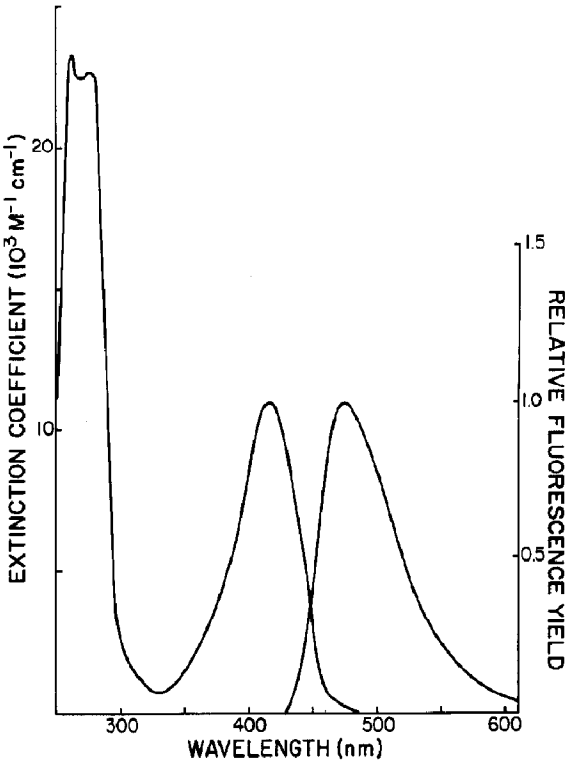

Fig. 2. Absorption (left) and fluorescence spectra of lumazine protein.

lumazine, Fig. 1) has a high fluorescence yield (0.58) and the fluorescence intensity decay is close to monoexponential, with lifetime of 14.7 ns (2°C) [8]. The steady state fluorescence anisotropy is high. Figure 3 is the decay of this anisotropy and after the excitation maximum, the data can be fitted to a single exponential function. For brevity in this review, the statistical justification for data fitting will be omitted and referred to the primary articles. Analysis of the data in Fig. 3 gives a rotational correlation time, $\phi = 20$ ns, which is in good correspondence to the value calculated from the Stokes–Einstein equation for the rotational diffusion of a 21-kD spherical macromolecule [8]. The sharp spike seen in the top panel of Fig. 3 before the excitation maximum, may be due to scattered light, although it is not evident at the higher resolution used in the lower panel. The data are very noisy in this time region because there is not much fluorescence signal.

It is also apparent that the bound lumazine does not exhibit any motion independent of the

rotation of the whole protein. Figure 3, bottom panel, is the anisotropy decay measured in the first few ns following the laser pulse excitation. Even within the 150-ps width of the instrumentally broadened laser pulse (dots), there is no suggestion of a rapid decay process, which would reflect a fast oscillatory type of motion of the lumazine within the protein's binding site. Again in the time region of the rise of the excitation pulse, the data are too noisy to extract what appears to be a ps-decay process. Figure 4 is a control measurement to show that in a mixture of both free and bound lumazine, the anisotropy decay of the rapidly rotating species, the free ligand (ϕ = 150 ps), is easily visualized.

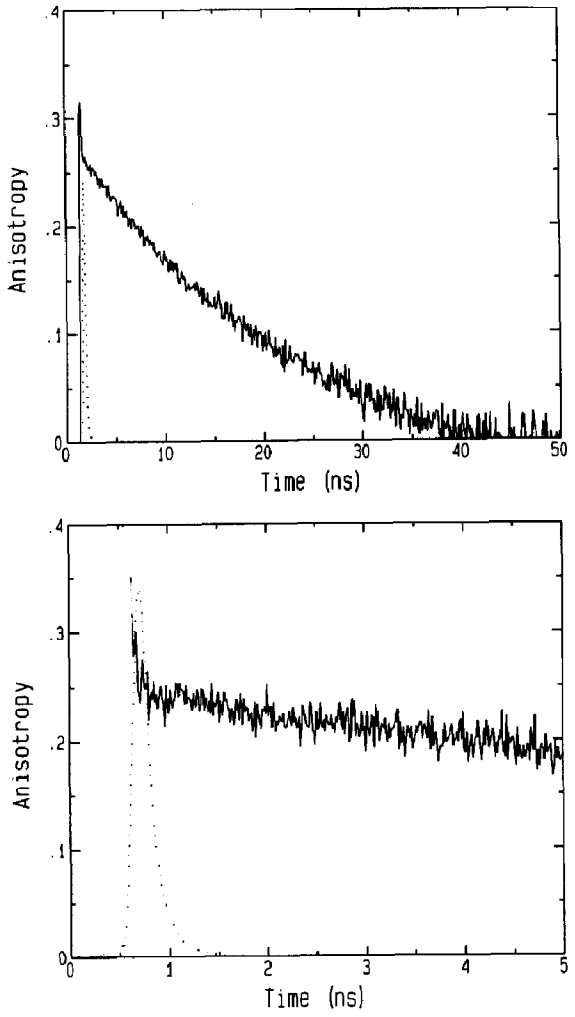

Fig. 3. Fluorescence anisotropy of lumazine protein (112 μM) from *Photobacterium phosphoreum*. Excitation 375, emission 475 nm, 0.05 *M* P_i, pH 7.0, 2°C. The dots represent the excitation pulse from the laser. The top and bottom panels are for different time scales.

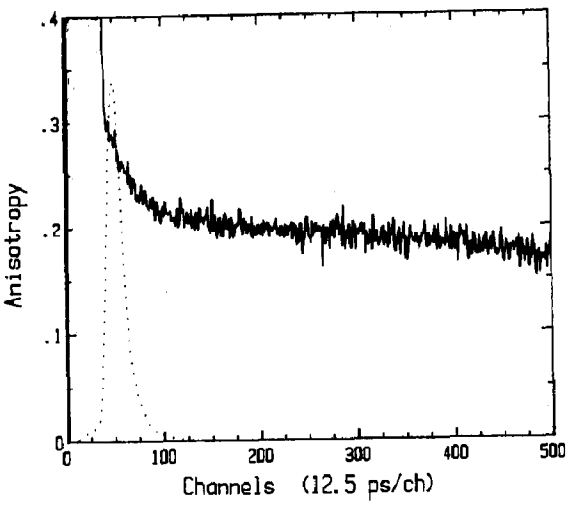

Fig. 4. Fluorescence anisotropy of a mixture of lumazine protein (20 μM) with the free lumazine derivative (20 μM). Other conditions are as in the legend of Fig. 3.

In aqueous solution the free lumazine derivative has a fluorescence maximum at 490 nm. This shifts to longer wavelength in solvents of lower polarity [9]. In a rigid medium at low temperature the fluorescence maximum for the analogous structure, 6,7,8-trimethyllumazine, is around 450 nm [10]. The fluorescence maximum at 475 nm for lumazine bound to the protein is therefore attributed to a highly polar environment being provided by the protein's binding site with the ligand immobilized. At the same time, evidence suggests that the site has a high surface exposure [9]. To achieve a high quantum yield of fluorescence it would be necessary for the lumazine's electronic ring system not to be in contact with amino acid residues that are potential fluorescence quenchers, viz., Trp, Tyr, Cys, and His. This requirement would be met and other properties of the bound ligand accounted for, if the lumazine attached itself by burying the ribityl group into the protein, with the lumazine electronic system exposed on the surface. Support for this suggestion is in the demonstrated importance of the side group's stereochemistry on the binding strength [11]. Other studies of fluorescence

quenching, circular dichroism, and NMR, are also consistent with these conclusions [11–13].

3. Protein–protein association

Bacterial luciferase has fluorescence around 330 nm from the Trp residues but nothing at longer wavelength. The fluorescence anisotropy decay of this Trp fluorescence or that of a bound extrinsic fluorophore such as ANS [6], both yield ϕ's in the range of 70 ns (2°C), consistent with the molecular mass of 76 kD for the luciferase $\alpha\beta$ dimer, and an approximately spherical shape.

If lumazine protein forms a complex with luciferase, the rotator to which the lumazine is attached is changed in mass from 21 kD to 97 kD. Visser and Lee [14] observed that, in the presence of certain types of luciferase, the average correlation time from the lumazine did indeed increase, an unambiguous demonstration of complexation. The fluorescence anisotropy decay of a mixture of lumazine protein and *Photobacterium leiognathi* luciferase is shown in Fig. 5 [15]. The decay cannot be fitted by a monoexponential function as in Fig. 3 for lumazine protein alone. In fact analysis requires a biexponential function for a statistically acceptable fit, with ϕ's of 20 and 100 ns, indicating the presence of two rotating species. From the analysis the K_d for the protein–protein dissociation can be recovered as 15 μM, in the range as would explain how lumazine protein could influence the bioluminescence at concentrations as low as 10 μM [5].

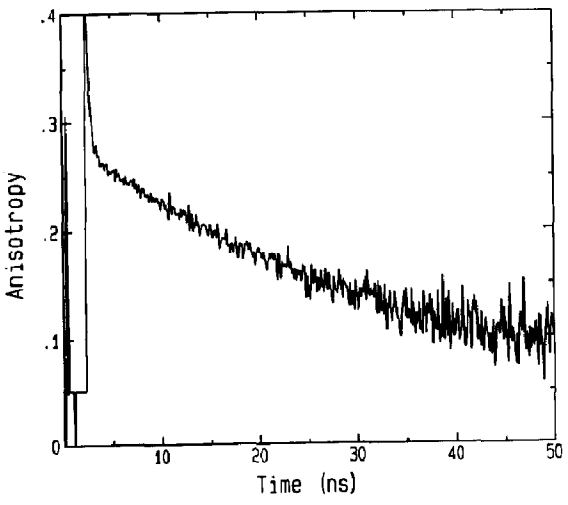

Fig. 5. Fluorescence anisotropy of a mixture of lumazine protein (20 μM) and luciferase (20 μM) both of the type *Photobacterium leiognathi*. Other conditions are as in the legend of Fig. 3.

However, in the case of *P. phosphoreum* luciferase, this explanation does not hold. The apparent equilibrium dissociation constant for this protein–protein dissociation is about 200 μM [15], whereas the bioluminescence effects are again almost saturated below a 10 μM concentration of lumazine protein. The idea has been propounded therefore, that lumazine protein forms a tighter complex with one of the metastable luciferase-bound flavin intermediate products mentioned above [15,16]. Consequently a study of the more relevant interaction with these intermediates was initiated.

4. Spectral properties of the luciferase intermediates

Figure 6 is the absorption spectrum of a luciferase peroxyflavin preparation [17]. The maximum in the 380 nm region is characteristic of dihydroflavin structures [18]. Preparations of luciferase peroxyflavin always exhibit weak fluorescence but this is mostly, if not entirely, attributable to impurities [2]. The absorption contribution around 450 nm in Fig. 6, is mostly accounted for by a small amount of the final product FMN, because fluorescence excitation at 450 nm produces a fluorescence distribution the same as FMN. Excitation at the 380-nm absorption maximum produces an additional fluorescence contribution at shorter wavelength around 490 nm due to a photochemical product impurity which is easily formed [19]. This "luciferase photoflavin" is a highly fluorescent species of unknown structure. It has similar spectral characteristics to the fluorescent transient but it does not have bioluminescence activity [2].

Addition of aldehyde to luciferase peroxyflavin yields the fluorescent transient [3,4,21] without change in the absorption spectrum [17]. The fluorescence maximum for the fluorescent transient

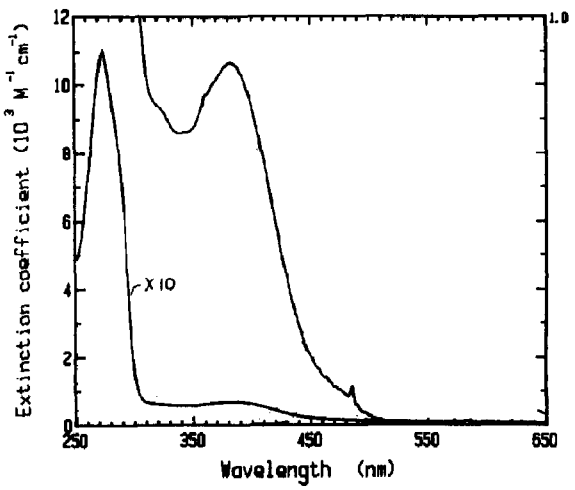

Fig. 6. Absorption spectrum of the *Photobacterium leiognathi* luciferase peroxyflavin; the left curve has been reduced X10 as indicated. Solution conditions are as in the legend of Fig. 3.

made from *P. leiognathi* luciferase, is at 496 nm and the spectrum corresponds exactly to the in vitro bioluminescence from this type of luciferase, evidence that the excited state of the fluorescent transient is also the origin of the bioluminescence emission. The fluorescence quantum yield is 0.33 and the decay of the fluorescence is practically

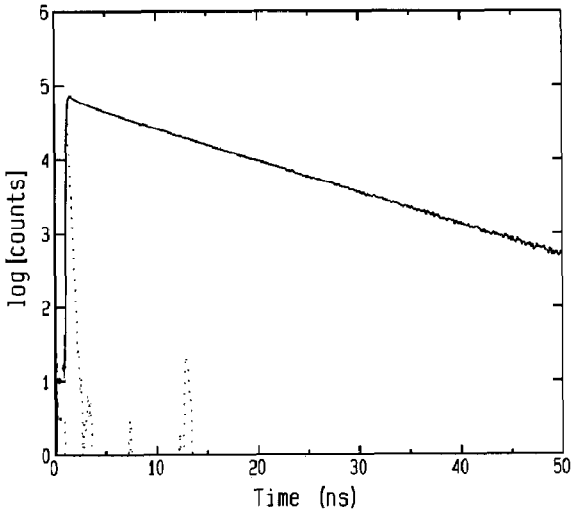

Fig. 7. Fluorescence of the *Photobacterium leiognathi* fluorescent transient. The dotted curve is the excitation pulse from the laser. Excitation 370 nm, emission 490 nm; other conditions are as in the Fig. 3 legend.

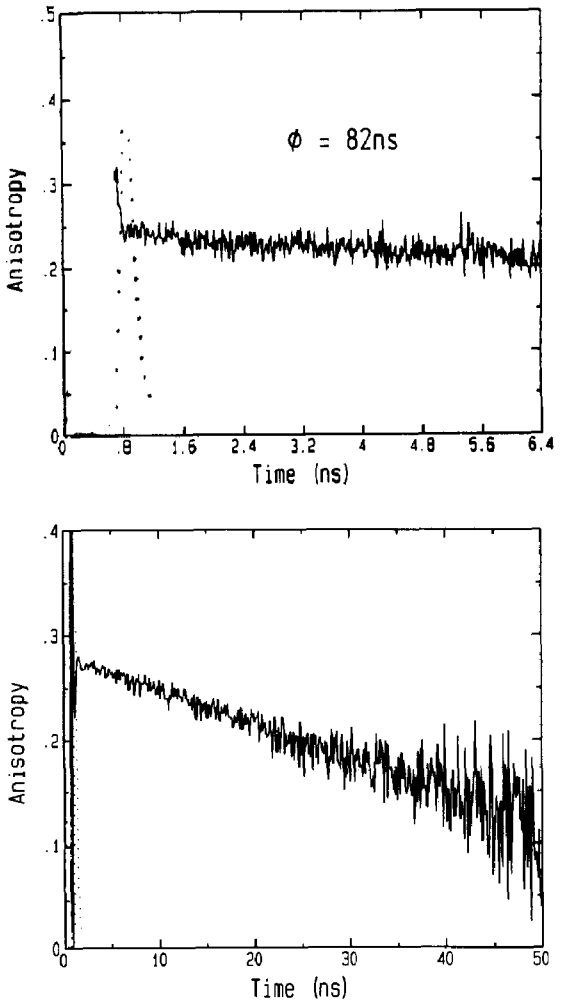

Fig. 8. Fluorescence anisotropy of the fluorescent transient (12 μM of the flavin chromophore). Excitation 377 nm, emission 470 nm, 0.05 M P_i, pH 7.0, 2°C, with tetradecanal and dodecanol added. The top and bottom panels are for different time scales.

monoexponential, with a lifetime of 10 ns (Fig. 7) [17].

This efficient fluorescence is unusual because flavoproteins are generally only weakly fluorescent, exceptions being flavins bound to apolumazine protein and the related Yellow Fluorescence Protein from *Vibrio fischeri* [12,20]. In free solution, however, FMN has a quantum yield of 0.25, with spectral maximum at 535 nm, and fluorescence lifetime of 4.7 ns. In free solution the fluorescence quantum yield of 1,5-dihydro-FMN ($FMNH_2$) is very small, but it is consider-

ably enhanced in rigid solvents and by some substituents at the 4a-position [21,22]. For reduced flavoproteins, the only fluorescent example is a substrate reduced derivative on lactate oxidase which exhibits an efficient fluorescence with a spectrum similar to that of the luciferase fluorescent transient [23].

Figure 8 shows the decay of the fluorescence anisotropy of the fluorescent transient. Global analysis was made of several sets of experiments taken over several hours, at channel widths of 12.5 ps and 100 ps, during which time the species is decaying to FMN. The fluorescence lifetime is 10.5 ns (88%) and the rotational correlation time is 82 ns, again in good correspondence with the Stokes–Einstein calculated value of rotational correlation time of luciferase.

The anisotropy decay measured at high time resolution in Fig. 8 (top), shows that there is no rapid loss of anisotropy within the 150 ps width of the laser excitation pulse [24]. In other words, like lumazine on its apoprotein, this luciferase-bound flavin fluorophore of the fluorescent transient has no motion independent of the rotation of the macromolecule.

5. Complexation with lumazine protein

Figure 9 shows the effect on the anisotropy decay when lumazine protein is added to a preparation of the fluorescent transient [24]. In contrast to Figs. 5 and 8, the fluorescence anisotropy of this mixture decays very rapidly ($\phi = 4$ ns). Fig. 9, bottom panel, shows no significant contribution of a second anisotropy decay component having the 23 ns correlation time expected for the added lumazine protein or the 82 ns correlation time from the luciferase-bound flavin. Global analysis of several experiments at 12.5 ps/channel and 100 ps/channel resolutions made over several hours, yield the parameters given in the Fig. 9 legend.

This rapid loss of anisotropy is concomitant with the shift of the bioluminescence spectrum that occurs when lumazine protein is included in the bioluminescence reaction with this luciferase. If lumazine protein is added to the reaction using

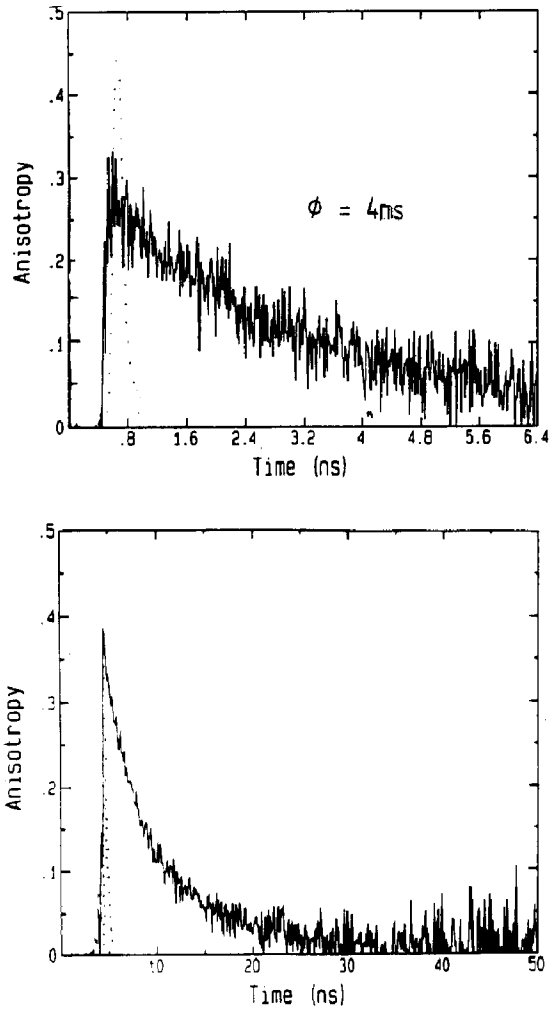

Fig. 9. Fluorescence anisotropy of a mixture of the fluorescent transient, initially 50 μM, and lumazine protein (20 μM). Excitation 370 nm, emission 460 nm; other conditions as in the legend of Fig. 8. The bottom panel shows that the fluorescence anisotropy measured out to 50 ns, shows no evidence of a longer correlation time. Global analysis of many data sets yields the parameters: $\tau_1 = 13.5$ ns (48%), $\tau_2 = 1.1$ ns (42%), $\tau_3 = 0.01$ ns, $r_0 = 0.292$, $\phi = 5.4$ ns.

instead the luciferase from *V. fischeri*, no bioluminescence spectral shift occurs and correspondingly, the fluorescent transient of *V. fischeri* luciferase does not show rapid fluorescence anisotropy loss when mixed with lumazine protein [17].

In the first attempt to detect complexation of lumazine protein with the luciferase intermediate species, for experimental reasons the *V. harveyi*

luciferase photoflavin was employed [16]. Although the anisotropy decay was found to be complex, the faster component had a $\phi = 25$ ns, not the strong effect expected and later observed with the *P. leiognathi* fluorescent transient. The belief that this photoflavin species was an appropriate model for the bioluminescence intermediate turned out to be incorrect. When instead the stabilised *V. harveyi* fluorescence transient was used this yielded the expected rapid rate of anisotropy loss [17], although not so dramatic as for *P. leiognathi*. This is consistent with the lesser amount of bioluminescence shifting when lumazine protein is added to the *V. harveyi* luciferase reaction [5]. The negative or weak results with the *Vibrio* luciferases make good controls for comparison to the strong effect of lumazine protein in the *Photobacterium* case.

This rapid decay of anisotropy is explained by energy transfer from the flavin donor on the luciferase (FT) to the lumazine while the two proteins are engaged in a protein–protein associated complex.

$$\mathrm{FT}^* \rightarrow \mathrm{FT} + 495\ \mathrm{nm} \qquad k_D$$

$$\mathrm{FT}^* \cdot \mathrm{LumP} \rightarrow \mathrm{FT} \cdot \mathrm{LumP}^* \qquad k_T$$

$$\mathrm{FT} \cdot \mathrm{LumP}^* \rightarrow \mathrm{FT} \cdot \mathrm{LumP} + 475\ \mathrm{nm} \qquad k_A$$

A measurement of the energy transfer rate k_T, was therefore attempted to give quantitative support to the idea that the same photophysical mechanism was involved in the bioluminescence.

6. Intraprotein energy transfer

Lumazine protein contains a single Trp residue located at the C-terminal [25]. Excitation into the absorption of this tryptophan residue produces two emissions, one with a maximum at 340 nm, and the other at 475 nm [9]. The 340 nm fluorescence is that usually observed for most tryptophan containing proteins and the 475 nm is from the bound lumazine. A distinct rise in the fluorescence at the longer wavelength has been measured and interpreted as due to energy transfer of the weak dipole coupling type, between the tryptophan as a donor and the lumazine as acceptor [12]. No rise is observed for the 340 nm fluorescence, nor for the 475 nm fluorescence if excited into the lumazine's lowest energy absorption band that has a maximum at 417 nm.

A systematic study of this energy transfer was made by varying the spectral properties of the bound ligand to change the spectral overlap and by using the range of excitation wavelengths conveniently available from a synchrotron source [26]. For excitation in the region around 300 nm and below, the fluorescence intensity from the lumazine was found to have both rise and fall components. The rise rate for lumazine protein was found to be 0.97 ns at 2°C, for excitation at 300 nm. From such data a separation between the tryptophan residue and the lumazine ligand was calculated to be about 2.7 nm. In a later study using a detection system with high time resolution (FWHM = 23 ps), it was found that these processes show complexity not previously realized from the lower resolutions used before [27].

7. The bioluminescence reaction

It has been proposed that the bioluminescence reaction produces the fluorescent transient as a primary excited species. If lumazine protein is present and in a protein–protein complex with the luciferase, this excitation could flow over to the lumazine ligand. However, the fluorescence maximum of the purported fluorescence donor is at 495 nm and the acceptor's absorption maximum is at 417 nm corresponding to a higher energy, and there is only a small spectral overlap (Fig. 2). Some have expressed the concern that this excitation transfer must be an "up-hill" process and therefore unlikely to be efficient. It has been proposed that there must be an alternative "high energy" primary excited state produced by the bioluminescence that would provide a larger spectral overlap. The following argument shows that this proposal is unnecessary.

For the fluorescence dynamics of weakly coupled electronic systems in homogeneous systems, the Förster equation [28] shows that the rate of energy transfer is governed not only by the extent of spectral overlap, J, but also by the mutual

orientation of the transition dipoles, represented by a parameter κ, and the average donor-acceptor separation, R.

$$k_T = k_D(R_0/R)^6 \quad (1)$$

where R_0 is called the critical Förster distance at which

$$k_T = k_D \quad (2)$$

and is given by

$$R_0^6 = (1.25 \times 10^{-25}) Q_F \kappa^2 J \quad (3)$$

The numerical value is the product of a number of constants, $Q_F = 0.33$, is the fluorescence quantum yield of the fluorescent transient donor and, for the purpose of calculation it can be assumed that the orientation factor is that for random collisions of donor and acceptor in free solution, which can be readily evaluated as, $\kappa^2 = \frac{2}{3}$. From Fig. 2 the spectral overlap is calculated as $J = 2 \times 10^{-15}\ M^{-1}\ \text{cm}^3$, which results in $R_0 = 22$ Å. For efficient deposition of the excitation onto the associated lumazine, we might set $k_T = 10\ k_D$, so that $R = 15$ Å. For the most favorable orientation, $\kappa^2 = 4$, the required separation only increases to 17 Å, because of the 6th power dependence.

Therefore in the lumazine protein–fluorescent transient complex, favorable orientation and proximity of the donor–acceptor pair could well outweigh the low value of spectral overlap and give rise to a highly efficient energy transfer.

Because 1-ns rise times were readily observable in the intra-protein experiments, reflecting energy transfer from the Trp residue to the bound lumazine, it was decided to make a similar direct measurement of the rate of energy transfer in the fluorescent transient–lumazine protein system, under the conditions exhibiting the fast anisotropy decay shown in Fig. 9 [29]. The results are in Fig. 10 with the analysis in Table 1. A rise contribution in the fluorescence function would be indicated by a negative amplitude for one of the exponentials but the analysis gives no statistical evidence for this. This is also qualitatively evident

Table 1

Fluorescence dynamics parameters for *Photobacterium* lumazine protein and the luciferase Fluorescent Transient

Time (h)	α_1 (%)	τ_1 (ns)	α_2 (%)	τ_2 (ns)	α_3 (%)	τ_3 (ns)	r_0	ϕ (ns)	Channel width (ps)
Lumazine protein									
	21	14.3	79	0.02			0.321	20.2	100
	40	*	60	*			0.278	*	12.5
	36	*	64	*			0.307	*	100
	30	*	70	*			0.223	*	12.5
	36	*	64	*			0.245	*	12.5
Fluorescent Transient									
0	22	10.4	72	0.005	6	0.3	0.282	82.2	12.5
0.8	25	*	68	*	7	*	0.373	*	12.5
2.6	15	*	81	*	4	*	0.307	*	12.5
3.1	9	*	90	*	1	*	0.289	*	100
3.9	55	*	25	*	20	*	0.330	*	12.5
Fluorescent Transient + Lumazine protein									
0	55	13.5	35	0.16	10	1.2	0.271	5.8	12.5
0.75	62	*	30	*	8	*	*	*	100
1.6	58	*	33	*	9	*	*	*	12.5
2.5	60	*	31	*	9	*	*	*	12.5
3.5	54	*	38	*	8	*	*	*	100
120	40	*	22	*	38	*	0.392	22	12.5

Excitation 377 nm, emission 460 nm, 2°C, 50 mM P_i; α, amplitude; τ, lifetime; r_0, initial anisotropy; ϕ, rotational correlation time; Time, is the number of hours after the preparation of the metastable fluorescent transient. For the global analysis, parallel and perpendicular data sets are combined for simultaneous fitting and * means that this parameter is linked in all sets.

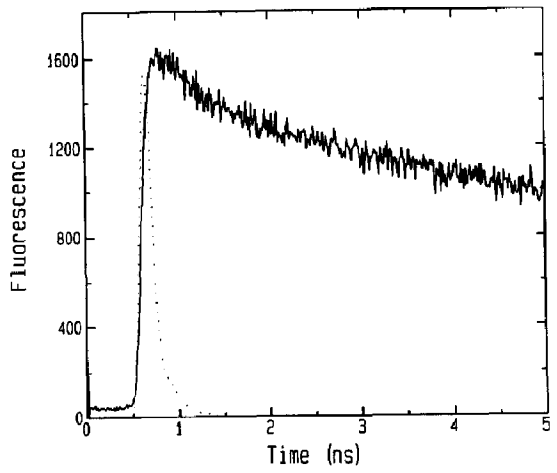

Fig. 10. Fluorescence of the sample measured in Fig. 9. Global analysis of several data sets is in Table 1.

in Fig. 10. If there is a rise of the putative lumazine acceptor fluorescence on excitation into the flavin absorption, it is hard to distinguish within the 150-ps (FWHM) resolution of this system. The rate of energy transfer is clearly quite fast.

The occurrence of a very rapid rate of energy transfer casts some doubt on the simple energy transfer scheme above, that is one of an initial excitation of the flavin followed by excitation transfer to the lumazine. It is reasonable to assume that the lumazine protein docks in a very specific way to the luciferase. Also from the high resolution anisotropy decay measurements (Figs. 3 and 8), it is evident that, within their respective proteins, there is a fixed positioning of the flavin and lumazine emission dipoles with respect to the protein axis of rotation. All these properties negate certain assumptions on which the Förster treatment is based, i.e., that diffusional averaging is a rate limiting factor [28].

Tanaka and Mataga [30] analyzed the fluorescence dynamics of a weakly coupled system that is a good model for the fluorescent transient-lumazine protein problem. In their approach they considered the donor-acceptor pair to be at a fixed separation and orientation. Assuming $k_T \gg k_D$ or k_A, the radiative rates of the donor and acceptor, respectively, the fluorescence of the acceptor is:

$$F_A(t) = r_D\left[3q_D - q_A\left(2\cos^2\alpha_{DA} + 1\right)\right]\exp(-k_T t) + q_A\left[r_D\left(2\cos^2\alpha_{DA} + 1\right) + 3r_A\right]\exp(-k_A t) \quad (4)$$

This is a simplified version of eq. (28) from Tanaka and Mataga [30], where r_D is the fraction of excitation light absorbed by the donor, and q_D the fraction of the emission, and the same for the acceptor. It is also assumed that the emission and absorption transition moments of the donor and of the acceptor, are coincident. The α_{DA} is the mutual orientation of donor and acceptor transition moment. At the concentrations used for the experiment shown in Fig. 10, 20 μM for lumazine and 50 μM for the flavin of the fluorescent transient, about 80% of the excitation at 370 nm is absorbed by the flavin and for the emission at 460, 80% is from the excited state of the lumazine. Substituting these values in eq. (4) yields:

$$F_A(t) = \left[0.8\times 3\times 0.2 - 0.8\times 0.8\left(2\cos^2\alpha_{DA} + 1\right)\right]\times\exp(-k_T t) + \left[0.8\times 0.8\left(2\cos^2\alpha_{DA} + 1\right) + 0.8\times 3\times 0.2\right]\times\exp(-k_A t) \quad (5)$$

Table 1 shows for the mixture, that the major fluorescence decay rate $(13.5\ \text{ns})^{-1}$ is slightly faster than the acceptor decay, $k_A = (14.3\ \text{ns})^{-1}$. This is attributable to admixture of the fluorescence decay from some unassociated fluorescent transient. The appearance of a negative amplitude in eq. (5), indicative of a rise rate, is seen from the equation to be critically dependent on the value of α_{DA}. Equation (5) predicts that a rise rate in the fluorescence will appear only for $\alpha_{DA} < 70°$. A negative amplitude of any significance does not come out of the global analysis in Table 1. There is, however, a significant increase in a fluorescence contribution having a 1 ns decay time in the mixture over the separate components. This decay rate of $1\ (\text{ns})^{-1}$ could plausibly

be equated to k_T. This would account for the shift in the bioluminescence by interaction with lumazine protein, because the radiative rate of the donor is only 0.1 $(ns)^{-1}$.

Tanaka and Mataga derived a very complex expression for the decay of emission anisotropy and showed that under the condition of no excitation of the acceptor and no emission detected from the donor, i.e., $r_A = q_D = 0$, the time dependence disappears. The conditions of the above experiment do not quite meet these same conditions because of the overlapping spectral properties of the donor and acceptor. The remaining time dependence is complex and not a simple exponential. The observed rapid loss of anisotropy results from certain combinations of the rate constants involved from which it is not possible to extract a quantitative estimate of k_T. It can be concluded, however, that the observed $\phi = 4$ ns in Fig. 9, is consistent with the 1 $(ns)^{-1}$ rate suggested for k_T.

An alternative to the time-dependent excitation transfer model is that in the complex the lumazine and the flavin form a cooperative electronic system. Excitation of the lumazine is simultaneous with that of the flavin, both in the fluorescence and bioluminescence process. Dipolar relaxation phenomena may also be important where the overlap integral for the initially excited flavin may be much greater than the static value. There is, however, no evidence for a ground state interaction of the donor–acceptor pair, but there are technical difficulties in making these measurements with good precision. The effect of changes in temperature and viscosity are clearly important to measure. An approach by using simulations especially on experiments with spectral variation of the ligands, will probably be a fruitful approach to these questions of the mechanisms of bioluminescence excitation and excitation transfer.

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References

1 J. Lee, I.B.C. Matheson, F. Müller, D.J. O'Kane, J. Vervoort and A.J.W.G. Visser, in: Chemistry and biochemistry of flavins and flavoproteins, ed. F. Müller, vol. II (CRC Press, Boca Raton, FL, 1991) pp. 109–151.
2 J. Lee, Y. Wang and B.G. Gibson, Anal. Biochem. 185 (1990) 220.
3 I.B.C. Matheson and J. Lee, Photochem. Photobiol. 38 (1983) 231.
4 M. Kurfürst, S. Ghisla and J.W. Hastings, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 2990.
5 J. Lee, Photochem. Photobiol. 36 (1982) 689.
6 J. Lee, D.J. O'Kane and B.G. Gibson, Biochemistry 27 (1988) 4862.
7 D.J. O'Kane and J. Lee, Methods Enzymol. 133 (1986) 149.
8 A.J.W.G. Visser and J. Lee, Biochemistry 19 (1980) 4366.
9 J. Lee, D.J. O'Kane and A.J.W.G. Visser, Biochemistry 24 (1985) 1476.
10 M. Sun, T.A. Moore and P.-S. Song, J. Am. Chem. Soc. 94 (1972) 1730.
11 J. Lee, B.G. Gibson, D.J. O'Kane, A. Kohnle and A. Bacher, Eur. J. Biochem. 210 (1992) 711.
12 T. Kulinski, A.J.W.G. Visser, D.J. O'Kane and J. Lee, Biochemistry 26 (1987) 540.
13 J. Vervoort, D.J. O'Kane, F. Müller, A. Bacher, G. Strobl and J. Lee, Biochemistry 29 (1990) 1823.
14 A.J.W.G. Visser and J. Lee, Biochemistry 21 (1982) 2218.
15 J. Lee, D.J. O'Kane and B.G. Gibson, Biophys. Chem. 33 (1989) 99.
16 J. Lee, D.J. O'Kane and B.G. Gibson, Biochemistry 28 (1989) 4263.
17 J. Lee, Y. Wang and B.G. Gibson, Biochemistry 30 (1991) 6825.
18 S. Ghisla, Methods Enzymol. 66 (1980) 360.
19 S.-C. Tu, Methods Enzymol. 133 (1986) 128.
20 S.C. Daubner, A.M. Astorga, G. Leisman and T.O. Baldwin, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 8912.
21 I.B.C. Matheson, J. Lee and F. Müller, Proc. Natl. Acad. Sci. U.S.A., 78 (1980) 948.
22 A.J.W.G. Visser, S. Ghisla and J. Lee, in: Flavins and Flavoproteins 1990, ed. B. Curti, S. Ronchi and G. Zanetti (Walter de Gruyter, Berlin, 1991) pp. 49–54.
23 S. Ghisla, Methods Enzymol. 66 (1980) 360.
24 J. Lee, E.A. Bradley and D.J. O'Kane, Proc. Soc. Photo-Opt. Instrum. Eng. 1640 (1992) 148.
25 D.J. O'Kane, B. Woodward, J. Lee and D.C. Prasher, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 1100.
26 A.J.W.G. Visser, A. van Hoek, D.J. O'Kane and J. Lee, Eur. Biophys. J., 17 (1989) 75.
27 J. Lee, G. Holtom and D.J. O'Kane, Proc. Soc. Photo-Opt. Instrum. Eng. 1204 (1990) 581.
28 T. Förster, Ann. Physik 2 (1948) 55.
29 J. Lee, Proc. Soc. Photo-Opt. Instrum. Eng. 1640 (1992) 542.
30 F. Tanaka and N. Mataga, Photochem. Photobiol. 29 (1979) 1091.