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Fluorescence Anisotropy Decay Study of Self-Association of Bacterial Luciferase Intermediates

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The fluorescence dynamics parameters of the fluorescent transient flavin-luciferase species from the types *Vibrio fischeri* and *Photobacterium leiognathi* are presented. The fluorescence anisotropy decay is a single exponential function for both types. The correlation time is 70 ns for the *P. leiognathi* fluorescent transient intermediate (2°C, aqueous buffer, pH 7.0), consistent with the rotational correlation time of the luciferase macromolecule (77 kD) to which the flavin fluorophore is rigidly attached. In contrast, for the *V. fischeri* species the observed correlation time for the anisotropy decay function is 133 ns. This suggests that protein self-association occurs in the *V. fischeri* case and this is confirmed by filtration, where the fluorescent transient from *V. fischeri* does not pass through a 100,000 molecular weight cutoff membrane, whereas the *P. leiognathi* species does. The filtration method also demonstrates self-association in the luciferase peroxyflavin and photoflavin from *V. fischeri.* A monomer-dimer equilibrium also explains the previously reported high correlation times for the *V. harveyi* luciferase-flavin species. It is proposed that the self-association competes with the lumazine protein interaction in the bioluminescence reaction.

KEY WORDS: Fluorescence anisotropy; rotational correlation time; protein association; bacterial luciferase; bioluminescence.

INTRODUCTION

The measurement of the rate of decay of fluorescence anisotropy is a valuable method for the study of hydrodynamics of macromolecules. For a spherical rotator the fluorescence anisotropy decay is a monoexponential function:

$$r(t) = r_0 \cdot \exp(-t/\phi) \quad (1)$$

where r_0 is the initial anisotropy and ϕ is the rotational correlation time. The rotational diffusion coefficient of a spherical macromolecule is given by the Debye–Stokes–Einstein equation:

$$D_s = kT/[6\eta V] \quad (2)$$

where k is the Boltzmann constant, T the absolute temperature, η the viscosity of the solution, and V the volume of the macromolecule. It can be shown that

$$\phi = (6D_s)^{-1} \quad (3)$$

Proteins can exhibit fluorescence from either intrinsic or extrinsic fluorophores. Intrinsic protein fluorescence almost always originates from the Trp residues, which absorb around 280 nm, with fluorescence maxima in the 310- to 350-nm range. There are many types of extrinsic fluorophores, both natural substrates and coenzymes, e.g., NADH or flavins, as well as artificial labels. Provided that these intrinsic or extrinsic fluorophores cannot move independently of the protein, i.e., their rotational diffusion is the same as the whole macromolecule, then their fluorescence anisotropy will reflect the motion of the whole macromolecule.

Proteins in general are not spherical and this com-

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plicates the interpretation of rotational correlation time measurements (for reviews see Refs. 1 and 2). For a symmetrical ellipsoid, not only will the rotational diffusion be different about the different axes but the ϕ's will also be a function of the angle subtended by the transition moment of the fluorophore with these axes. Generally these complications lead to a measured ϕ which is larger than from Eqs. (2) and (3) for an equivalent sphere, ϕ_s [2]. For example, in the case of lumazine protein, which has an axial ratio of 1.3, the theoretical model for the anisotropy decay approximates to a three-exponential [3]:

$$r(t) = \beta_1 \cdot \exp(-t/[1.097\phi_s]) + \beta_2 \cdot \exp(-t/[1.061\phi_s]) + \beta_3 \cdot \exp(-t/[0.964\phi_s]) \quad (4)$$

In an experimental measurement of lumazine protein, the statistics of counting does not allow its $r(t)$ function to be distinguished from a single exponential [4]. In measurements of the fluorescence dynamics of macromolecules, the extraction of more than two ϕ's is usually feasible only if they differ substantially.

This paper is concerned with the interpretation of the results of fluorescence dynamics measurements of some fluorescent species formed by reaction of reduced flavin ($FMNH_2$) with the bacterial luciferases from two distinct types of the marine bioluminescent bacteria, *Vibrio* and *Photobacterium*. All bacterial luciferases react with $FMNH_2$, O_2, and tetradecanal, to produce bioluminescence emission in a broad spectrum, with maximum in the range 487–505 nm, depending on the type of bacterium from which the luciferase was obtained [5]. In the course of the reaction a fluorescent transient species forms having the same fluorescence spectrum as the bioluminescence, so it is evident that the bioluminescence must originate from the fluorescent state of this same fluorescent transient molecule [6,7].

A metastable luciferase peroxyflavin can be produced if the aldehyde is left out of the reaction above [8, 9]. The addition of aldehyde to this peroxy form then produces bioluminescence along with the fluorescent transient, so it is believed that this peroxy species is an intermediate in the bioluminescence process [7]. Luciferase peroxyflavin is only weakly fluorescent, however [10], but it can be photochemically converted to a highly fluorescent form, the luciferase photoflavin, which has fluorescence properties very similar to those of the fluorescent transient, except that the fluorescence maximum is at about a 10-nm-longer wavelength [11]. All these luciferase-flavin products may be stabilized for several hours, long enough for the measurement of their fluorescence dynamics properties [12].

Luciferase in its native state, however, shows only intrinsic fluorescence. In the case of the luciferase from *V. harveyi*, which has 8 Trp residues, the ϕ has been measured using either the intrinsic fluorescence (ϕ = 62 ns) or an extrinsic fluorophore, 8-anilino-1-naphthalenesulfonic acid (ANS; ϕ = 74 ns), at 2°C and in 50 m*M* P_i [12]. From Eqs. (2) and (3),

$$\phi_s = M_r(\bar{v} + h)/(RT) \quad (5)$$

and luciferase has a molecular weight, M_r = 77,000, calculated partial specific volume, $\bar{v}$ = 0.73 cm^3/g, and hydration, h = 0.35 cm^3/g. Then the calculated value is ϕ_s = 61 ns (2°C). If it is supposed that the transition moments of the 8 Trp's might be more or less randomly oriented, this would explain why the intrinsic ϕ value averages to the near-spherical one. A low-resolution X-ray structure for luciferase indicates an axial ratio of about 1.5 [13]. Only one ANS molecule is bound to luciferase [14]. If luciferase in solution rotates as a prolate ellipsoid with this same axial ratio, then the ANS transition moment and the longer axis would have to be nearly parallel to account for its ϕ = 74 ns [12].

In contrast, however, all the fluorescent *V. harveyi* luciferase-flavin reaction species described above show a significantly higher ϕ, about 100 ns [12]. Two explanations can be advanced for this high value, the first being that going to these intermediate states, the luciferase undergoes a gross conformation change to an axial ratio >2.2, with the single bound flavin's transition moment parallel to the long axis. The second possibility is that the luciferase self-associates in these intermediate states. An equilibrium mixture of monomer and dimer would result in an average ϕ lower than the one calculated for the dimer [Eq. (5), 122 ns]. In support of the monomer–dimer equilibrium explanation, it was observed that the fluorescence yield of the luciferase photoflavin decreased on dilution, which would result if the dimer had a much higher fluorescence yield than the monomer. However, independent approaches to the molecular weight measurements, i.e., ultracentrifugation and light scattering, although not conclusive, did not favor the molecular weight increase as explaining the anomalous value of ϕ [12].

Two technical advances now allow us to resolve this question in favor of self-association. A rapid method of ultrafiltration is available with a cutoff at a molecular weight of 100,000 so that the luciferase monomer can quickly be separated from the dimer, in a few minutes. The intermediates from two other types of luciferase, *V.*

fischeri and *Photobacterium leiognathi*, have been prepared with a sufficient stability for precise fluorescence dynamics investigation. The fluorescent transient of *P. leiognathi* luciferase passes through the filter and its ϕ = 70 ns, consistent with the M_r = 77 kD, the same as native luciferase. The intermediates from *V. fischeri*, on the other hand, are retained largely on the filter and have ϕ = 133 ns. The high values found for ϕ are therefore due to self-association in the case of the *Vibrio* luciferase-flavin species.

MATERIALS AND METHODS

The luciferases were purified to highest specific activity from the bioluminescent bacteria types *V. fischeri* strain "Pony" [11] and *P. leiognathi* strain S1 [15, 16]. Luciferase concentrations were assayed by absorbance assuming that the ϵ(280 nm) = 85,000 M^{-1} cm^{-1} for each type [17]. 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* [12], where it was called the "low-fluorescence intermediate II." 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 using a Centrex Centrifugal Microfilter (Schleicher and Schell, Keene, NH) as previously described. The absorbance spectrum of a 1.0-ml luciferase peroxyflavin fraction was first measured. The fluorescent transient was prepared by the immediate addition of 1 μl of tetradecanal saturated in ethanol. The absorbance spectrum was then rerun. 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. The luciferase photoflavins were prepared simply by irradiation of the peroxyflavin as previously described [12]. Spectra were determined with a Hewlett–Packard 8452A Diode Array Spectrophotometer with the sample at 5°C. The concentration of the luciferase intermediate was estimated from the absorbance at the maximum near 380 nm. The flavin derivative is approximately stoichiometric with the luciferase and the extinction coefficient is ~10^4 M^{-1} cm^{-1}.

Filtration was carried out at 2°C in an Amicon Model 3 stirred cell (Amicon, Danvers, MA) fitted with a YM-100 membrane, nominal molecular weight cutoff 100,000. The sample of volume, usually 2 ml, was introduced into the upper compartment of the cell and concentrated to 1 ml in less than 5 min by applying a nitrogen pressure of 60 psi. The filtrate and retentate were clarified by centrifugation for 5 min in an Eppendorf Microfuge, Model 5412, before measuring the absorbance spectra.

Emission decay measurements were made with a laser system and single photon counting electronics described in detail elsewhere [18]. Emission lifetime and anisotropy decay parameters were determined from the experimental vertical and horizontal fluorescence data sets by the rigorous method of deconvolution from the laser pulse set (FWHM, $\leq$0.2 ns) and simultaneous fitting of the vertical, I_v, and horizontal, I_h, deconvolved data sets to the two equations [19],

$$3I_v = S(t) \cdot [1 + 2r(t)] \quad (6)$$

$$3g \cdot I_h = S(t) \cdot [1 - r(t)] \quad (7)$$

where the total fluorescence intensity is given by the sum

$$S(t) = I_v + 2g \cdot I_h \quad (8)$$

and the time-dependent anisotropy is

$$r(t) = D(t)/S(t) \quad (9)$$

where

$$D(t) = I_v - g \cdot I_h \quad (10)$$

and g is the "g-factor" correcting the response of the system to horizontally polarized emission to that of vertically polarized emission [18].

For the fitting the software routine LIFETIME, generously provided by Dr. Gary R. Holtom (Pacific North-West Laboratories, Richland, WA), was used. In fitting most of the data the sums of exponentials models are assumed [20]:

$$S(t) = \Sigma_i \alpha_i \cdot \exp(-t/\tau_i) \quad (11)$$

$$r(t) = \Sigma_j \beta_j \cdot \exp(-t/\phi_j) \quad (12)$$

with i = 3 or 4 and j = 1 or 2; τ is the fluorescence lifetime, ϕ is the rotational correlation time, and α and β are the respective amplitudes.

RESULTS

Figure 1 compares the fluorescence anisotropy decay of the flavin derivative associated with the luciferase fluorescent transient prepared from *V. fischeri* (top) and

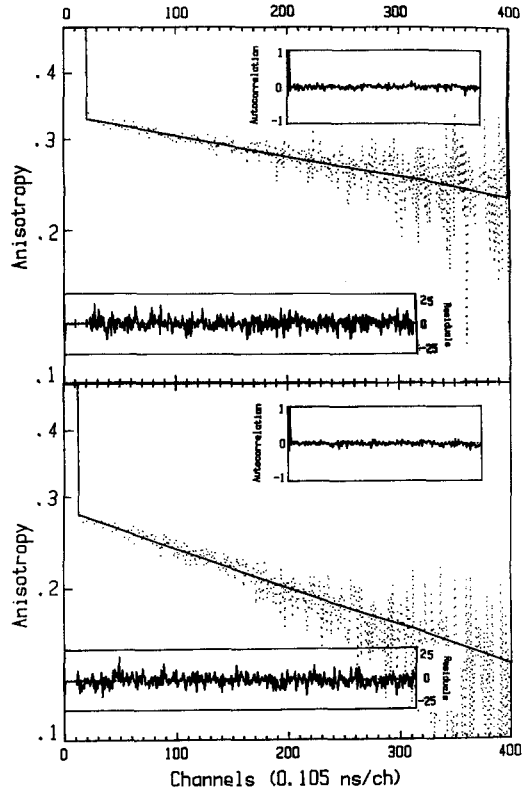

Fig. 1. Emission anisotropy decay (log scale) of the luciferase fluorescent transients. The points are the data and the lines the function based on the parameters in Table I. The excellent quality of the fits is shown by the insets, the weighted residuals and autocorrelation. The excitation pulse (FWHM <0.2 ns) was 370 nm and emission 460 nm, the slit width was 20 nm, and the sample was at 2°C. Top: *V. fischeri* luciferase. Bottom: *P. leiognathi* luciferase.

Table I. Decay Parameters for the Fluorescence and Anisotropy of Luciferase Fluorescent Transients[a]

Fluorescence						Anisotropy		
α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	β_1	ϕ_1 (ns)	χ^2
V. fischeri								
0.55	10.0	0.09	2.5	0.36	0.27	0.33	133	1.24
(0.01)[b]	(0.02)	(0.002)	(0.2)	(0.02)	(0.01)	(0.001)	(6)	
P. leiognathi								
0.62	10.5	0.11	1.8	0.26	0.2	0.28	70	1.17
(0.001)	(0.01)	(0.003)	(0.04)	(0.01)	(0.01)	(0.001)	(0.2)	

[a]Excitation, 370 nm; emission, 460 nm; 2°C, 50 m*M* P_i; pH 7.0.
[b]SD (standard deviation for the fit) given in parentheses.

P. leiognathi (bottom). The fluorescence dynamics parameters are given in Table I and the solid lines in Fig. 1 are functions constructed using these parameters. The points are the data and it can be judged from the residuals plot, which is randomly around zero, and the autocorrelation plots, which show no oscillation, as well as the near-unity value of χ^2 in Table I, that the fits are excellent [20]. It is apparent in Fig. 1 that the slopes of the decays in the two panels are quite different.

These data are from a measurement within the first hour after adding tetradecanal to the luciferase peroxyflavin. The analysis of data sets taken at later times gives the same result for the dominant long fluorescence lifetime, about 10 ns, and ϕ in the range 133–156 ns for *V. fischeri* and 70–77 ns for *P. leiognathi.* Because the ϕ/τ ratio is >10 in the former case, less confidence can be placed in the absolute value of ϕ [4]. Nevertheless, the conclusion can be safely stated that the ϕ's for these two types of luciferase fluorescent transients differ in magnitude about two times. Also, it is seen in the data that the initial anisotropy for the *P. leiognathi* intermediate, $\beta = 0.28$, is less than for the *V. fischeri* inter-

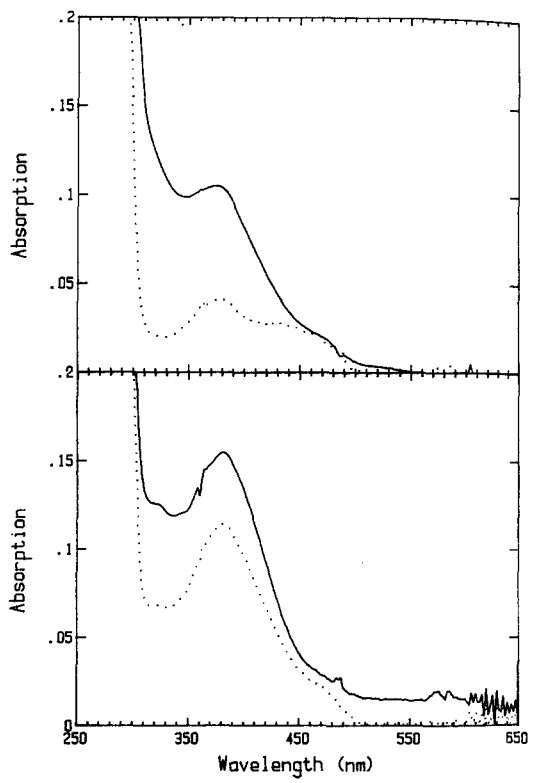

Fig. 2. Absorption spectra of the luciferase fluorescent transient intermediates before and after filtration through the 100,000 molecular weight cutoff membrane. The solid line is the original solution, and the dotted line the filtrate (2°C). Top: *V. fischeri,* 33 μ*M* originally. Bottom: *P. leiognathi,* 43 μ*M* originally.

mediate, 0.33. This would arise if the bound flavin possessed a degree of fast oscillatory motion in its binding site on the *P. leiognathi* protein.

In Fig. 2 are the absorption spectra of the luciferase fluorescent transients before the filtration (solid line) and of the filtrate. The maximum near 380 nm is of the luciferase-bound flavin and is characteristic of a dihydroflavin electronic structure [21]. The small shoulder in the region of 450 nm is due mostly to the FMN decomposition product from the intermediate [12]. The initial volume in this case was 3 ml, and 1 ml of the filtrate was obtained. In the top panel in Fig. 2 (*V. fischeri*), the major part of the 380-nm absorbance, especially after allowing for the FMN contribution, is retained by the filter, indicating that it has a molecular weight in excess of 100,000. It is seen that the FMN-like absorption is passed mostly by the filter. In contrast, in the bottom panel in Fig. 2 (*P. leiognathi*), almost all the 380-nm absorbance passes through the filter, indicating a molecular weight <100,000. For a control measurement a solution of native *V. fischeri* luciferase of the same concentration completely passed through the membrane.

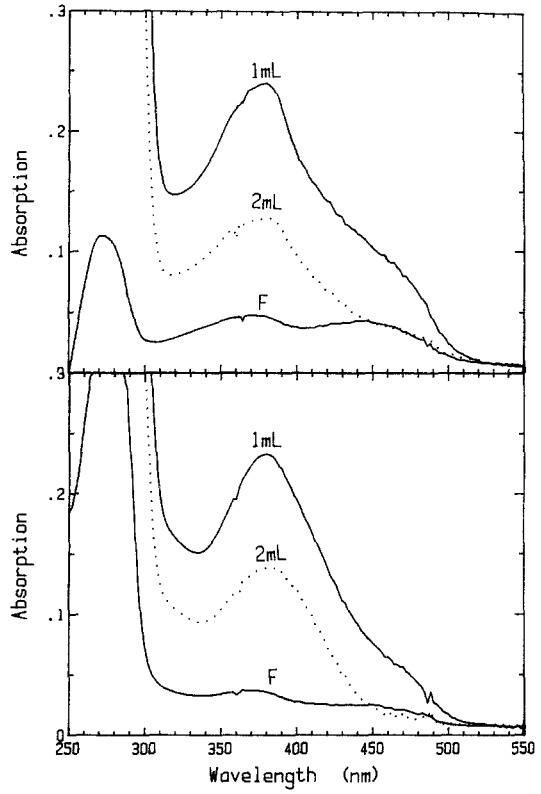

Fig. 3. Absorption spectra of *V. fischeri* luciferase-flavin species showing that they do not pass the 100,000 molecular weight membrane. The original solution (dotted line), 2-ml volume, is concentrated to 1 ml (solid line), and the spectrum of this retentate rerun. F is the filtrate. Top: Luciferase peroxyflavin. Bottom: Luciferase photoflavin.

In Fig. 3 are the results of filtration experiments on the *V. fischeri* peroxyflavin (bottom) and the photoflavin (top). In both panels it is quite clear that the 380-nm absorbing species are not passed by the 100-kD cutoff filter. By noting that the ratio of 450-to-370 nm absorbance is large, most of the absorbance in the filtrate can be attributed to FMN, either free or bound to luciferase. All of the luciferase intermediate species have dihydroflavin-like absorbance spectra with negligible contribution in the 450-nm region.

The peroxyflavins are only weakly fluorescent and so reliable parameters are not available for these. In the case of the photoflavin from this strain of *V. fischeri*, the fluorescence dynamics data have been published before [11]. Again, there is a dominant fluorescence lifetime of 9.4 ns and the ϕ = 142 ns with a standard deviation of 11 ns for five different samples.

DISCUSSION

The question can be asked whether the self-association of the *Vibrio* luciferase intermediates has any mechanistic significance. There are several relevant arguments to consider. Bacterial luciferase is made up of two nonidentical subunits, α and β, and in the native state exists in the unassociated $\alpha\beta$ = 77 kD form in solution [22]. Recently using genetic techniques, a form of *V. harveyi* luciferase was constructed with the α and β subunits fused together [23]. This fused luciferase exhibited a degree of self-association ($\alpha_2\beta_2$) but without change in the specific activity for the bioluminescence reaction. This means that self-association is not rate limiting in the bioluminescence.

Matheson and Lee [6] suggested that the fluorescent transient could function as a sensitizer or acceptor of the bioluminescence excitation in a manner similar to the function of lumazine protein. They proposed this model to explain the complex kinetics of bioluminescence at high, but physiologically relevant, concentrations of native *V. harveyi* luciferase. This acceptor function would require that the two flavins in the associated dimer be close enough together for energy transfer to occur between them, say below 20 Å, depending on the orientation factor of the Förster equation, or even closer if electron transfer were involved [24].

Energy transfer usually provides a route of anisotropy loss [25] decreasing the ϕ markedly, as observed in the case of the *V. harveyi* intermediates interacting

with lumazine protein [26, 27]. The value of ϕ of 133 ns for *V. fischeri*, however, is close to that calculated for a spherical dimer. In the luciferase-flavin dimer, if the electronic transition moments of the flavin pair were parallel, fast energy transfer could occur without any anisotropy loss being observed. At the other extreme, if the two flavins are close but the transition moments are orthogonal, the rate of energy transfer will be very slow and the value of ϕ, again, hardly affected. Therefore on the question of the proximity of the flavins, no conclusions can be reached.

The ϕ around 100 ns observed for the *V. harveyi* case [12] is intermediate between that of the unassociated luciferase as exemplified by the *P. leiognathi* intermediates and the self-associated *V. fischeri* intermediates. If the *V. fischeri* intermediates are more fully dimerized than the *V. harveyi* intermediates, this explains why the standard methods for molecular weight determination failed to reveal higher molecular weight species in the *V. harveyi* experiments. The methods took too long and the intermediates decayed, or they involved dilution (chromatography) and dissociation took place.

It can be proposed that in order for the lumazine protein to affect the bioluminescence reaction, acting as an acceptor for the bioluminescence energy with the resultant spectral shift and quantum yield increase, the lumazine protein has to compete with the self-association reaction. This is based on the fact that the strength of the bioluminescence effect is inversely correlated with the extent of self-association. The effect of lumazine protein can be observed at a few micromolar concentration for the *in vitro* bioluminescence reaction of *P. leiognathi* luciferase, at about a 10 times higher concentration for *V. harveyi*, and not, or hardly, at all for *V. fischeri* [28].

It is possible also to dismiss these ideas about the mechanistic significance of the self-association of the intermediates. Certainly this would be the case if the two flavins of the dimer were not proximate, accounting also for the failure to observe energy transfer between them. On the other hand, the properties of the bioluminescence reaction are well known to depend on the bacterial types, the *Vibrio* and the *Photobacterium* types being the most well studied. There is also a variability introduced with aldehyde chain length, although this does not seem to be a factor in the dimerization since the peroxyflavin of *V. fischeri* also shows a molecular weight higher than 100,000. Another factor to note is that lumazine protein is found only in bacteria of type *Photobacterium.* The fluorescent transient appears to be the *in vivo* bioluminescence emitter used in all the *Vibrio* types except for the one type called strain Y1 of *V. fischeri*, which uses a fluorescent flavoprotein as its emitter [29–31]. This "yellow fluorescent protein" has a primary structure strongly homologous to that of lumazine protein [32].

It will be useful to plan experiments to compare some of the bioluminescence properties of the types of luciferase to see if the concept of self-association might be useful in explaining differences in kinetics, substrate quantum yields, stoichiometry, and lumazine protein effects.

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REFERENCES

1. L. Brand, J. R. Knutson, L. Davenport, J. M. Beechem, R. E. Dale, D. G. Walbridge, and A. A. Kowalczyk (1985) in P. M. Bayley and R. E. Dale (Eds.), *Spectroscopy and the Dynamics of Molecular Biological Systems*, Academic Press, London, pp. 259–305.
2. G. R. Fleming (1986) *Chemical Applications of Ultrafast Spectroscopy*, Oxford University Press, New York.
3. A. J. W. G. Visser, A. van Hoek, D. J. O'Kane, and J. Lee (1989) *Eur. Biophys. J.* **17,** 75–85.
4. A. J. W. G. Visser, T. Ykema, A. van Hoek, D. J. O'Kane, and J. Lee (1985) *Biochemistry* **24,** 1489–1496.
5. J. Lee (1985) in J. G. Burr (Ed.), *Chemi- and Bioluminescence*, Marcel Dekker, New York, pp. 401–437.
6. I. B. C. Matheson and J. Lee (1983) *Photochem. Photobiol.* **38,** 231.
7. M. Kurfürst, S. Ghisla, and J. W. Hastings (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2990–2994.
8. J. W. Hastings and Q. H. Gibson (1963) *J. Biol. Chem.* **238,** 2537.
9. J. Vervoort, F. Muller, J. Lee, W. A. M. van der Berg, and C. T. W. Moonen (1986) *Biochemistry* **25,** 8062–8067.
10. C. Balny and J. W. Hastings (1975) *Biochemistry* **14,** 4719.
11. J. Lee, Y. Wang, and B. G. Gibson (1990) *Anal. Biochem.* **185,** 220–229.
12. J. Lee, D. J. O'Kane, and B. G. Gibson (1988) *Biochemistry* **27,** 4862.
13. R. F. Swanson, L. H. Weaver, S. J. Remington, B. Matthews, and T. O. Baldwin (1985) *J. Biol. Chem.* **260,** 1287–1288.
14. S.-J. Tu and J. W. Hastings (1975) *Biochemistry* **14,** 4310–4316.
15. D. J. O'Kane and J. Lee (1985) *Biochemistry* **24,** 1467.
16. D. J. O'Kane and J. Lee (1986) *Methods Enzymol.* **133,** 149.
17. D. J. O'Kane, M. Ahmad, I. B. C. Matheson, and J. Lee (1986) *Methods Enzymol.* **133,** 109–128.
18. J. Lee, D. J. O'Kane, and B. G. Gibson (1989) *Biophys. Chem.* **33,** 99–111.
19. A. J. Cross and G. R. Fleming (1984) *Biophys. J.* **46,** 45–46.
20. D. V. O'Connor and D. Phillips (1984) *Time Correlated Single Photon Counting*, Academic Press, London.
21. F. Müller (1983) *Topics Curr. Chem.* **108,** 71–107.

22. M. M. Ziegler and T. O. Baldwin (1981) *Curr. Top. Bioenerg.* **12,** 65–113.
23. A. Escher, D. J. O'Kane, J. Lee, and A. A. Szalay (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6528–6532.
24. J. Lee (1990) in H.-C. Curtius, S. Ghisla, and N. Blau (Eds.), *Pteridines and Folic Acid Derivatives,* Walter de Gruyter, Berlin, pp. 445–456.
25. M. Fayet and Ph. Wahl (1969) *Biochim. Biophys. Acta* **181,** 373–380.
26. J. Lee, D. J. O'Kane, and B. G. Gibson (1989) *Biochemistry* **28,** 4263.
27. J. Lee, Y. Wang, B. G. Gibson, and D. J. O'Kane (1990) *Proc. Soc. Photo-Opt. Instr. Eng.* **1204,** 706–716.
28. J. Lee (1982) *Photochem. Photobiol.* **36,** 689.
29. E. G. Ruby and K. H. Nealson (1977) *Science* **196,** 432–434.
30. S. C. Daubner, A. M. Astorga, G. B. Leisman, and T. O. Baldwin (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8912–8916.
31. P. Macheroux, K. U. Schmidt, P. Steinerstauch, S. Ghisla, P. Colepicolo, R. Buntic, and J. W. Hastings (1987) *Biochem. Biophys. Res. Commun.* **146,** 101–106.
32. D. J. O'Kane, B. Woodward, J. Lee, and D. Prasher (1991) *Proc. Natl. Acad. Sci. USA,* in press.