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Dynamic fluorescence study of the interaction of lumazine protein with bacterial luciferases

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The equilibrium association of lumazine protein from *Photobacterium phosphoreum* with luciferases from either *P. phosphoreum* or an aldehyde-requiring dark mutant of *Vibrio harveyi* is measured from changes of the rotational correlation time which is derived from the decay of the lumazine ligand's fluorescence anisotropy. The rotational correlation time of lumazine protein is 23 ns (2°C, 0.25 M P_i) and is increased on addition of luciferase due to the formation of a higher molecular weight complex. The *V. harveyi* luciferase exhibits full competence for the association and a 1:1 stoichiometry, with a K_d in the range 40–90 μM. At lower ionic strength (0.05 M P_i), the K_d increases but is reduced again by the addition of dodecanol or dimyristoyllecithin. In contrast, tetradecanal, a substrate for the bioluminescence reaction, exerts no influence on the association. The equilibration rate is found to be too slow and for both luciferases the K_d values are too high for the interaction of the native proteins to account quantitatively for the spectral shifting of the bioluminescence by lumazine protein.

1. Introduction

Lumazine protein is a 21.2 kDa protein containing 6,7-dimethyl-8-ribityllumazine as a noncovalently bound prosthetic group (for recent reviews, see ref. 1). Lumazine protein is isolated from *Photobacterium* where it functions as the antenna for the emission of the bioluminescence. Evidence for this role includes the effect on the bioluminescence spectrum from the in vitro reaction of bacterial luciferase. The emission maximum around 495 nm, from the reaction of the substrates $FMNH_2$, O_2 and aldehyde with luciferase, is shifted to 475 nm on inclusion of lumazine protein, and the bioluminescence spectral distribution matches that of the fluorescence of the lumazine protein itself. This bioluminescence shifting effect takes place at protein concentrations around 10 μM and it was proposed therefore that a protein-protein interaction was involved in the mechanism. Visser and Lee [2] measured the rotational correlation time (ϕ) of lumazine protein (LumP) from the decay of the fluorescence anisotropy of the bound lumazine and showed that ϕ was increased in the presence of luciferase (E), a direct demonstration of complexation between these two proteins. They proposed an equilibrium:

$$E + LumP \Leftrightarrow E \cdot LumP \quad (1)$$

For the free lumazine protein, $\phi = 23$ ns (2°C) and for the complex having a molecular weight about 5-times greater, they suggested that ϕ should be found to be around 75 ns. By an analysis of the anisotropy decay function and its dependence on concentration they were able to extract the thermodynamic parameters. In the case of the luciferase from *Vibrio harveyi* and lumazine protein from *P. phosphoreum*, they estimated the dis-

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sociation constant to be $K_d = 2$ μM (2°C, 50 mM P_i, pH 7.0). For the luciferase from *P. phosphoreum*, however, the binding was much weaker (about 1 mM) and this was not an expected result, since only 10 μM lumazine protein produced a substantial spectral shift for both types of luciferases [3].

It is the intention of this present work to make a more systematic study of this binding equilibrium with particular regard to the question of the binding competence of luciferase. The question of competence is a general one which arises in protein physical chemistry. Is a protein purified to homogeneity by the standard criteria, also functionally homogeneous, e.g., for an enzyme activity or a protein-protein interaction? It is a question which is usually experimentally difficult to answer and therefore often ignored. This problem did arise, however, in the work of Visser and Lee [2] where it was found that more than 50% of the *V. harveyi* luciferase did not have binding competence for the lumazine protein, in spite of the fact that the preparation was thought to have the highest bioluminescence activity and was certainly homogeneous by the usual protein criteria. Homogeneity of the preparation must be established first of course, before we can extract meaningful equilibrium thermodynamic parameters from the observations.

In this work we will show that the study of the anisotropy decay of the luciferase-lumazine protein system has the potential for accurate and precise recovery of the equilibrium parameters. A more systematic investigation than that carried out previously is now feasible, since methods have been developed for production of both required proteins in quantity and of much higher bioluminescence activity in the case of the luciferases [4,5]. The luciferase preparation from *V. harveyi* is shown to be homogeneous for the binding equilibrium, thus removing a major uncertainty in the previous determination of the properties of this protein association. What is also revealed by this study, however, is that there are factors of unknown origin which produce variability in binding and do not allow the accuracy of the technique to be fully realized. The conclusion is reached that the bioluminescence properties of lumazine protein are not strongly correlated with the parameters of its association with luciferase in the native state.

2. Materials and methods

Bacterial luciferases were from *V. harveyi* strain MAVA, an aldehyde-deficient dark mutant, and from *P. phosphoreum*, strain A13. The luciferases were purified to homogeneity and assayed for bioluminescence activity as described elsewhere [4]. The luciferases used for this study had specific bioluminescence activities of at least 75% of the maximum observed. This compromise on activity was made in order to obtain sufficient amounts of protein to cover all conditions as completely as possible. Lumazine protein was from *P. phosphoreum* strain A13, and was purified to spectroscopic homogeneity as described by O'Kane and Lee [5]. Protein concentrations were assayed by absorbance; for the luciferases, $\epsilon(280) = 85000$ $M^{-1}\,cm^{-1}$; for lumazine protein, $\epsilon(417) = 10100$ $M^{-1}\,cm^{-1}$ [6]. Bovine immunoglobulin G (IgG) was from Sigma (St. Louis, MO; 99%) and 8-anilino-1-naphthalenesulfonic acid (ANS) was a gift from Dr. J. Brewer. All other chemicals were of the best grades that could be obtained commercially.

Emission decay measurements were made with excitation from a laser system and detection by single-photon counting electronics. The laser system was from Spectra-Physics (Mountain View, CA) and consisted of a mode-locked Nd/YAG laser, frequency-doubled, synchronously pumping a dye laser containing the dye LDS 751 (Exciton Chemical, Dayton, OH). The lasing wavelength was set at either 770 or 796 nm depending on the measurement. The dye laser output was intracavity-dumped to produce single pulses of about 15 ps width at a rate of 800 kHz or 4 MHz, with an average power of 45–200 mW. These pulses were frequency-doubled to the excitation wavelength, 385 or 398 nm, using an angle-tuned KDP or $LiIO_3$ crystal. The output after doubling was horizontally polarized, so the polarization was returned to the vertical with a Soleil-Babinet compensator, subsequently passed through a CS 4-96

(Corning) color filter to remove the 770 nm feed-through from the dye laser, then through a Glan-Thompson prism and into the sample cuvette. This cuvette was contained in a temperature-controlled housing. The complete optical system was purchased from Applied Photophysics (London, U.K.). The fluorescence from the sample passed through a Glan-Thompson prism then through 4-97 and either 3-73 or 3-74 color filters; the latter two completely eliminate the scattered excitation. In all experiments the stray light from the samples was measured and found to be negligible. The fluorescence radiation passed into an $f/3.4$ grating monochromator, with a grating having 1200 lines/mm blazed at 500 nm. Slits of 5–23 nm width were used depending on the signal strength and no influence on the final result was noted. The signal was detected at the exit slit by a three-stage microchannel plate photomultiplier (Hamamatsu 2287U) operated at −3750 V. The output was sent to a standard single-photon counting electronics system for processing.

If the sample cuvette contained a scattering solution of dilute glycogen the 'prompt curve' could be obtained by removing the 3-73 or 3-74 color filter and setting the emission monochromator to the same wavelength as the excitation. The prompt curve was collected before and after each sample measurement. It had a full-width at half-maximum of less than 200 ps. The excitation intensity was adjusted so that the total count rate for the collection of the prompt curve was about the same as for the sample; for most measurements this was in the range 3–8 kHz. No 'color shift' [7] of the prompt curve was detected, an important advantage of the use of a microchannel plate photomultiplier. The data were accumulated in 512 channels of a 1024-channel multichannel analyzer. The channel width was calibrated with delay cables and was kept at 0.140 ns/channel for all measurements. At the end of the counting period, the data were transferred to a computer (DEC 11/73) for processing. Standard samples having single fluorescence lifetimes were used to verify the performance of the system and the literature values were recovered [7].

For each measurement two decay curves were collected. With the polarization of the exciting pulses first set to the vertical, the emission polarizer was set to the vertical position and counts collected for 10 s and dumped into the first half of the multichannel analyzer. Then the emission polarizer was automatically turned through 90° and the 'perpendicular' decay curve collected for 10 s, and stored in the second half of the multichannel analyzer. This process was repeated usually more than 50 times until a sufficient number of counts were collected, about 2×10^4 in the peak channel. The sum of the data sets collected in the parallel position is referred to as 'PAR' and the perpendicular sets 'PER'. Because of the L-configuration and particularly due to the use of the monochromator, an accurate G factor must be determined. This was performed in essentially the same way as for steady-state anisotropy measurements [8]. For the sample under study after determination of PAR and PER, the Soleil-Babinet compensator and excitation polarizer were rotated to the horizontal positions and a new set of decay curves G(PAR) and G(PER) collected in the same way as described above, from which the G factor was determined as, $G = G(\mathrm{PAR})/G(\mathrm{PER})$, averaged from the channel containing the maximum number of counts out to about channel 400, to avoid highly scattered values.

The time-dependent 'experimental' anisotropy is given by

$$R(t) = (\mathrm{PAR} - G \cdot \mathrm{PER})/F(t), \tag{2}$$

where $F(t)$ is the total experimental fluorescence decay:

$$F(t) = \mathrm{PAR} + 2G \cdot \mathrm{PER}. \tag{3}$$

The analysis of decay curves is by weighted least-squares deconvolution and this is a well-established procedure [7]. The analysis routines extract the prompt function from the experimental decays to obtain the 'true' decays, $r(t)$ and $f(t)$, which are then fitted to the sums of exponential processes:

$$r(t) = \sum_i \beta_i \exp(-t/\phi_i) \tag{4}$$

where ϕ_i is a rotational correlation time, and

$$f(t) = \sum_i \alpha_i \exp(-t/\tau_i) \tag{5}$$

where τ_i is a fluorescence decay time and the pre-exponential factors are amplitudes of the individual processes, and are summed to unity, or to 100 in the tables. For the $r(t)$ function the amplitudes are scaled:

$$r_0 = \sum_i \beta_i, \tag{6}$$

where r_0 is the initial anisotropy.

Fitting programs from two independent sources were employed. The first source was from Applied Photophysics and these routines were speedy, robust and had the convenience of running on the 11/73 computer in the laboratory. With these routines the analysis time is competitive with the data collection time. The second source was through the generosity of A.J.W.G. Visser [9]. These programs had more versatility than those from Applied Photophysics and were used in modified form to run on a Cyber 850 mainframe computer. The fitting programs from either source were statistically rigorous when applied to the analysis of $F(t)$ curves and yielded essentially the same results. For an acceptable result, $\chi^2 < 1.20$ usually, with a Durbin-Watson parameter (DW) greater than the value appropriate for the number of exponential terms in the model: for $i = 1$, DW > 1.7; $i = 2$, DW > 1.75; $i = 3$, DW > 1.8 [7].

For the analysis of anisotropy decay the routine from Applied Photophysics is oversimplified in that it deconvolutes eq. 2 and fits $r(t)$ by a least-squares procedure [7]. In spite of this approximate procedure, the results, at least when knowing the correct decay model, are not very different from the rigorous global procedure of ref. 9. The global procedure is more cumbersome to use but yields meaningful values of the statistical parameters. Most of the correlation times in the tables were obtained using the Applied Photophysics routine, with checks made at appropriate points using the global method. All decay curves were fitted from the channel (except where indicated otherwise) corresponding to the zero time, taken to be the maximum of the prompt pulse, to some channel in the region 430–480, to cut off the highly scattered data.

3. Results

The decay of the fluorescence anisotropy of lumazine protein is retarded in the presence of luciferase. This was first shown by Visser and Lee [2] who presented results similar to those demonstrated in fig. 1. The data here show two features not previously observed. Because of the higher resolution of the present measuring system, a rapidly decaying component in the anisotropy decay can be clearly seen at early times in both the free lumazine protein (top) and the complex (lower). The source of this rapid loss of anisotropy could be from some of the free ligand in equilibrium with bound or from a 'wobble in the cone' motion of the bound, but it is not accurately measured in these present experiments. A more informative study of this fast motion would re-

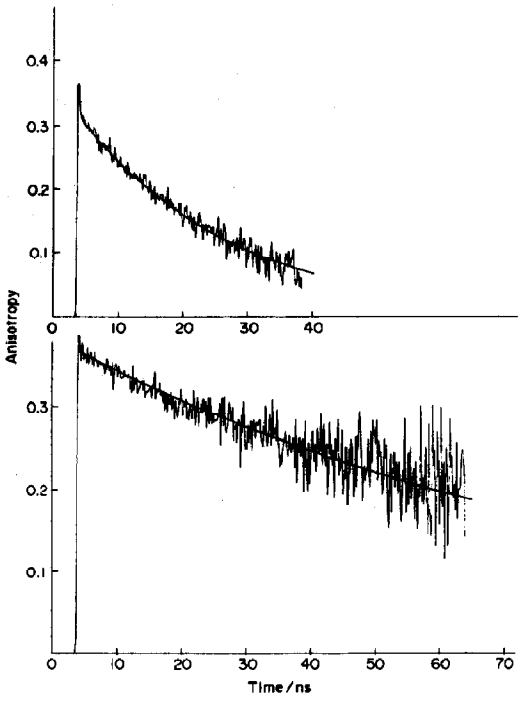

Fig. 1. Decay of emission anisotropy. The line is the fit to the data. (Top) Lumazine protein (20 μM); $r(t) = 0.04 \exp(-t/0.2) + 0.31 \exp(-t/23.5)$; $\chi^2 = 1.2$, DW = 1.70. (Bottom) Complexed with *V. harveyi* luciferase (100 μM); $r(t) = 0.05 \exp(-t/0.2) + 0.34 \exp(-t/91.1)$; $\chi^2 = 1.15$, DW = 1.79. λ_{ex} 385 nm, λ_{cm} 475 nm; 0.25 M P_i, pH 7.0, 2° C.

quire a higher resolution than used in these present data sets. Of current concern is that, according to statistical criteria the anisotropy data, even starting from $t = 0$, can be fitted to a single-exponential decay in most cases. For example, in fig. 1 the data in the top panel also fit the function, $r(t) = 0.32 \exp(-t/22.9)$ with $\chi^2 = 1.15$ and DW = 1.69 (time values are in ns), and for the lower panel the single-exponential fit is $r(t) = 0.34 \exp(-t/91.8)$, with $\chi^2 = 1.19$ and DW = 1.74. These statistically acceptable fits result because of the overwhelming weight of the data at the longer times. Since this shorter component is visually present and its neglect biases the correlation time to slightly lower values, all results reported here are from a two-exponential-decay model.

The second result arising from fig. 1, also in table 1, is that the correlation time of the lumazine protein in the presence of high concentrations of *V. harveyi* luciferase is greater than the 75 ns observed with similar concentrations by Visser and Lee [2]. However, the luciferase used here was isolated from the aldehyde-requiring mutant of *V. harveyi* rather than the wild type used previously and, since the present luciferase also exhibits a much higher bioluminescence activity than the wild type [5], it might be expected that it shows a greater degree of competence for binding lumazine protein and is more fully associated at the same luciferase protein concentration.

It was noted by Visser and Lee [2] that the correlation time derived from the decay of the bound lumazine's fluorescence anisotropy indicated that the lumazine was rigidly attached to the apoprotein, i.e., it rotated along with the whole protein. This is the time of 23.5 ns in fig. 1. The rotational correlation time of a protein is related to the molecular weight, M_r, the viscosity of solution, η, absolute temperature, T, partial specific volume, $\bar{v}$, and degree of hydration, h, by the Stokes-Einstein equation:

$$\phi = M_r \eta (\bar{v} + h)/RT. \quad (7)$$

Fig. 2 is presented as a control study to demonstrate that eq. 7 is a good approximation for some proteins and some cases of deviation. In addition to lumazine protein, luciferase and aldolase with the bound extrinsic probe ANS which has a complex fluorescence decay with a major lifetime component of about 15 ns, as well as bovine serum albumin (BSA) with ANS and luciferase measured by its intrinsic fluorescence [10], all give $\phi T/\eta$ independent of temperature. Also, in fig. 2 it can be seen that the ratio of ϕ values at 2°C for lumazine protein, luciferase, and aldolase, 1 : 3.6 : 7.4, is in good correspondence with that expected from eq. 7, 1 : 3.2 : 8.1.

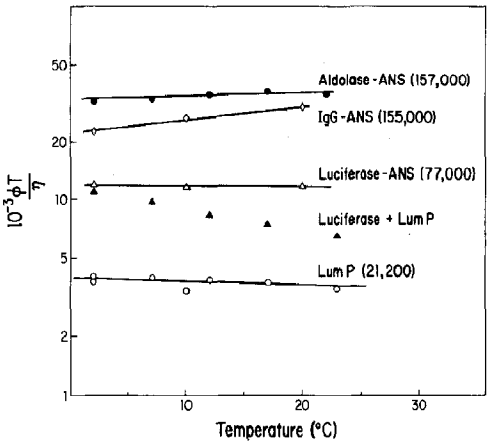

Fig. 2. The Stokes-Einstein equation, $\phi = \eta M_r(\bar{v} + h)/RT$, for calculation of the rotational correlation time of a spherical protein, is obeyed for lumazine protein (20 μM) and *V. harveyi* luciferase ($A_{280} = 16$) but not for their complex (luciferase + LumP), due to its temperature-induced dissociation. λ_{ex} 385 nm, λ_{em} 475 nm. M_r values given in parentheses. All in 0.25 M P_i, pH 7.0, 2°C.

Eq. 7 does not hold for ANS bound to IgG. This protein is elongated and undergoes structural changes with temperature, responsible for the increase in ϕ [11,12]. The mixture of lumazine protein and luciferase giving the lengthened value of ϕ (fig. 1) also deviates but in the opposite direction to IgG. Although high concentrations of protein could increase the viscosity of the solution slightly, this could not account for the anomalous behavior of this mixture. The simple explanation is that increase in temperature dissociates the protein-protein complex.

Tables 1 and 2 contain the results from analysis of the fluorescence dynamics of the bound lumazine ligand in lumazine protein-luciferase mixtures. The solutions contained 0.25 M P_i, since high ionic strength lowers the dissociation con-

Table 1

Emission decay parameters for mixtures of lumazine protein and *V. harveyi* luciferase (0.25 M P_i)

Conditions: 2° C, pH 7.0; λ_{ex} 385–398 nm, λ_{em} 475 nm; $\alpha_1 + \alpha_2 = 100$; ϕ_{av} is the longer correlation time from an unconstrained two-exponential fit to the anisotropy decay: $r(t) = \beta_1 \exp(-t/\phi_1) + \beta_2 \exp(-t/\phi_2)$. [E] (luciferase) and [LumP] (lumazine protein) denote initial concentrations.

Row	[E] (μM)	[LumP] (μM)	Fluorescence				Anisotropy	
			α_1	τ_1 (ns)	α_2	τ_2 (ns)	ϕ_{av} (ns)	β_1/β_2 [a]
1	0	20	92	14.7	8	1	25.2	
2	20		88	14.4	12	0.5	48.7	1.95
3	50		94	14.1	6	1	67.2	0.66
4	100		88	13.8	12	1	84.4	0.32
5	175		94	13.5	6	2	99.8	0.19
6	360		91	13.6	8	1	110	0.13
7	42	5	90	13.4	10	1	72	0.39
8		10	90	14.2	10	3	71	0.40
9		20	88	14.3	12	4	63.4	0.51
10		30	90	14.3	10	4	55.5	0.79
11		60	92	14.3	8	3	42.4	1.35
12		83	93	14.5	7	3	42.5	1.30
13	93 [b]	20	84	13.6	16	3	94	0.17

[a] From an analysis starting from $t \sim 0.5$ ns with the ϕ values constrained: $r(t) = \beta_1 \exp(-t/25.2) + \beta_2 \exp(-t/123)$.
[b] Luciferase of 3-fold lower bioluminescence specific activity.

Table 2

Titration of luciferase and lumazine protein from measurement of the decay of lumazine's emission anisotropy

Conditions: 2° C, pH 7.0, 0.25 M P_i; λ_{ex} 385 nm, λ_{em} 475 nm; β_1/β_2 is obtained from a fit to the decay of the anisotropy, starting from $t \sim 0.5$ ns and with the ϕ values constrained: $r(t) = \beta_1 \exp(-t/22) + \beta_2 \exp(-t/74)$.

[E] (μM)	[LumP] (μM)	Correlation times (ns)				Average (ns)	β_1/β_2	S.D.	% complex
42	20	41	43	40	36				
		39	34			38.8	0.98	0.25	51
52.5		39	40	39		39.3	0.90	0.07	53
105		44	47			45.5	0.58		63
127		46	48			47	0.54		65
176.5		53	52	54		53	0.36	0.03	74
210		51	59	51	52	53.3	0.33	0.08	75
417		61	70	58	58				
		70	66			63.8	0.12	0.07	89
42	40	34	35	38	37	36.0	1.17	0.18	44
110	5	58	58			58	0.22		3.7
	10	52	55			53.5	0.32		6.9
	20	46	47			46.5	0.53		11.9
	30	46				46	0.56		17.5
	50	48	47			47.5	0.50		30
	100	45	47			46	0.56		58
	150	39				39	0.94		70

stant [2], thus minimizing the amounts of protein required for a systematic study. Each result is the average of two to four measurements of a sample and in table 1, rows 1–6 are averages of the results from three or more samples, and the remaining rows are for single samples. In table 2, where there are three or more samples the standard deviation of the sample average is reported. The results are divided into two, tables 1 and 2, because they were from preparations of luciferases that showed differences in binding properties, in spite of their having the same bioluminescence activity.

There is not much effect on the fluorescence decay when lumazine protein associates with luciferase. The previous observation of a small reduction in τ_1 accompanying the binding [2] is confirmed in table 1 (rows 1–6). Along with the increase in ϕ_{av} there is an accompanying decrease in τ_1, amounting to 1 ns on full association. The small amplitude of the shorter fluorescence lifetime component, 0.5–2 ns, does not permit it to be estimated very accurately. There is also a small increase in the initial anisotropy on binding, shown typically in fig. 1. For free lumazine protein the initial anisotropy (i.e., at $t = 0$) averages around 0.33, increasing slightly in the complex to 0.36.

It was pointed out for fig. 1 that the anisotropy decay contains a short component of rather uncertain value. Only the longer correlation time, labelled ϕ_{av}, is listed in the tables. The β_1/β_2 column in tables 1 and 2 is the amplitude ratio from an analysis of the anisotropy decay with the

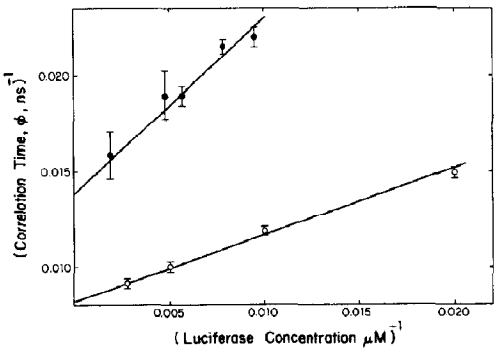

Fig. 3. Rotational correlation time of lumazine protein (20 μM) plotted vs. concentration of *V. harveyi* luciferase. Results are from two different preparations of luciferase (see tables 1 and 2); λ_{ex} 380, λ_{em} 475 nm, 0.25 M P_i, 2°C.

ϕ values constrained to the values corresponding to free and luciferase-bound lumazine protein, as determined below. This β_1/β_2 ratio represents the ratio of the concentrations of free over bound lumazine protein. As pointed out by Visser and Lee [2], this is reasonable because the absorption and fluorescence spectra of the bound lumazine do not change in the luciferase-lumazine protein complex. The only spectral property observed to be affected is the fluorescence decay lifetime, having a less than 10% drop (table 1). This may result in only a slightly lower fluorescence quantum yield of the associated lumazine protein and can be neglected. Then, if we consider ϕ_{av} to be a simple average from the free and bound lumazine proteins:

$$(\beta_1 + \beta_2)\phi_{av} = \beta_1\phi_1 + \beta_2\phi_2 \quad (8)$$

$$\beta_1/\beta_2 = (\phi_2 - \phi_{av})/(\phi_{av} - \phi_1). \quad (9)$$

If eq. 1 holds we can write, where K is the equilibrium constant and square brackets denote concentrations:

$$K = [E][LumP]/[E \cdot LumP] \quad (10)$$

$$= [E]\beta_1/\beta_2 \quad (11)$$

and then

$$(\phi_{av} - \phi_1)^{-1} = [E]^{-1}K(\phi_2 - \phi_1)^{-1} + (\phi_2 - \phi_1)^{-1} \quad (12)$$

Fig. 3 contains reciprocal plots of ϕ_{av} vs. the total luciferase concentration, with the lumazine protein concentration held constant. The lower points are from table 1, rows 1–6, the upper being from the first group of table 2. Eq. 12 shows that as $[E]^{-1}$ approaches zero, ϕ_{av} approaches the value of ϕ_2. In the range chosen the points are linear and the lines are obtained by regression analysis. For the lower line, $r = 0.99$, and from the intercept we can estimate ϕ_2(bound) to be 123 ns, with a standard deviation of 4 ns. The upper data plotted from table 2 yield a lower estimate for ϕ_2(bound) of 74 ns, with standard deviation 3 ns and $r = 0.92$.

Rows 7–12 of table 1 and the second group in table 2 ([E] = 110 μM and below) are a reverse titration keeping [E] constant and the behavior of ϕ_{av} is seen to be consistent with the equilibrium

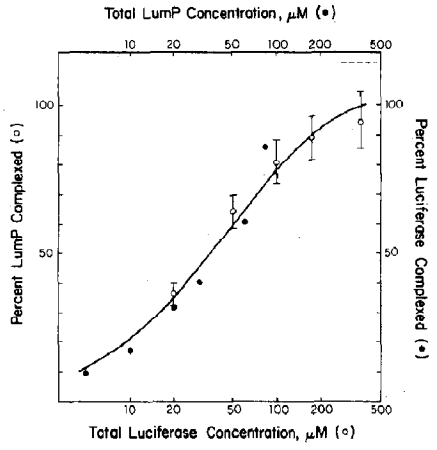

Fig. 4. Binding curve for *V. harveyi* luciferase as a titrant vs. lumazine protein (LumP), 20 μM (○), or lumazine protein as a titrant vs. luciferase, 42 μM (●). Data are from table 1 and the error bars are standard deviations. The full line is a least-squares fit of all the points to the equation: $C = 112[T]/(44 + [T])$, where [T] is the total titrant concentration. The dashed line at the top right is at 112%.

model, also confirming again that any small increase in solution viscosity due to protein concentration does not affect these measurements significantly.

Finally, in table 1 (row 13), it is shown that a luciferase preparation of low specific activity binds the lumazine protein just as effectively as that of the highest specific activity, as judged from the value of ϕ_{av}. The lower activity of this luciferase preparation was consequent to its reaction with $FMNH_2$ and O_2 (from intermediate II preparation [10]), so it can be concluded that whatever causes this activity loss does not reduce the lumazine protein-binding effectiveness.

Figs. 4 and 5 are binding plots from the data of tables 1 and 2, respectively. In fig. 4, the results of adding increasing concentrations of luciferase to a constant concentration of lumazine protein are distinguished from the reverse titration. In the former case, the percent complex, C, is simply obtained from eq. 10 as:

$$C = (1 + \beta_1/\beta_2)^{-1}. \qquad (13)$$

For the reverse titration the percent luciferase complexed to lumazine protein is expressed as:

$$C = [\mathrm{LumP}]_T/\left([E]_T(1 + \beta_1/\beta_2)^{-1}\right), \qquad (14)$$

where the subscript T denotes the total concentration, i.e., bound plus free. The titrant concentrations are seen to be sufficiently high to exceed the point of inflexion in the binding curves and this justifies fitting the data to the binding equation:

$$C = C_M[T]/(K + [T]), \qquad (15)$$

where [T] represents the total concentration of titrant, K the equilibrium constant, and C_M the maximum percent complex, 100% for the 1:1 stoichiometry implied by eq. 1.

The full lines in figs. 4 and 5 are obtained by fitting the points to eq. 15 by a least-squares method using a routine based on the Marquardt algorithm and provided by Dr. J.E. Wampler, University of Georgia. The parameters for the fit are reported in the figure legends and show a 2-fold difference in the equilibrium constants between the two luciferases. Both sets of data, however, are satisfactorily fitted by a C_M near to 100%, i.e., there is no evidence that the stoichiometry is not 1:1 (eq. 1) or that the luciferase is less than 100% competent (within rather large uncertainty, however). On this point table 2 shows that more than 70% of the luciferase is complexed when the lumazine protein titrant is at 150 μM. We did not use higher concentrations than this because of the possibility of distortion due to the high absorbance of the solution. Therefore, we

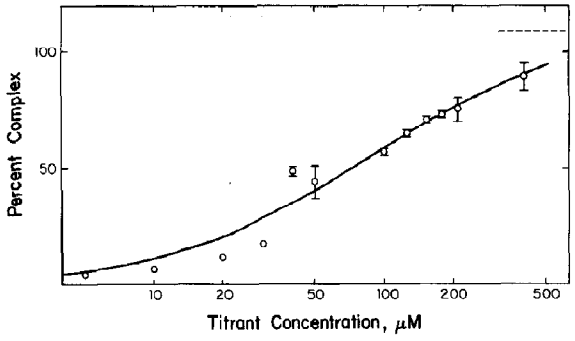

Fig. 5. Binding curve of a different *V. harveyi* luciferase preparation from that in fig. 4. Data are listed in table 2. All points are combined and where there are three or more samples, the standard deviations are given, represented by the error bars. For luciferase as a titrant, the lumazine protein is 20 μM; for lumazine protein as titrant, the luciferase is 110 μM. The least-squares fit is the equation: $C = 109[T]/(89 + [T])$.

Table 3

Interaction of lumazine protein and luciferases: kinetics and effect of additives

Conditions: 2° C, pH 7.0; rows 1–8, λ_{ex} 398 nm, λ_{em} 470 nm; rows 9–26, λ_{ex} 385 nm, λ_{em} 475 nm. Luciferase from *V. harveyi* (rows 1–21) and from *P. phosphoreum* (rows 22–26).

Row	[E] (μM)	[LumP] (μM)	ϕ_{av} (ns)	Conditions
1	20	20	35.5	3 min (rows 1–8, 0.25 M P_i)
2			34.9	12 min
3			40.5	25 min
4			42.7	38 min
5	50	20	49.6	2 min
6			56.8	6 min
7			57.6	12 min
8			61.6	30 min
9	0	20–120	23.3 [a]	(rows 9–26, 0.05 M P_i)
10	42	30	38.0	10 μM dodecanol
11	42	30	41.2	20 μM dodecanol
12	39	29	44.9	39 μM dodecanol
13	39	28	53.3	84 μM dodecanol
14	36	26	53.1	164 μM dodecanol
15	32	23	50.3	294 μM dodecanol
16	32	23	51.8	294 μM dodecanol + 24 h
17	42	20	52.8	100 μM dodecanol
18	42	20	42.9	
19	42	20	41.2	85 μM tetradecanal
20			39.1	85 μM tetradecanal
21			48.5	85 μM lecithin
22	53	20	28.3	
23			29.7	25 μM dodecanol
24			29.1	25 μM dodecanol + 2 days
25			29.3	50 μM dodecanol
26			32.4	100 μM dodecanol

[a] S.D. 0.9 ns.

cannot reliably estimate the saturation value of the luciferase complexation taking the lumazine protein titrant data alone. These data, however, are consistent with those from the reverse titration and together result in the parameters reported in the legends.

The results in table 3 were from the same luciferase preparation as used for table 1. These are averages of single samples. The first sets of observations (rows 1–8) show that there is a measurable equilibration time for the protein-protein association. In these experiments the emission slit widths were 23 nm so as to have a count rate of about 25 kHz, enabling decay curves with good statistics to be collected in times as short as 2 min. The time listed under the Conditions column is the mid-time for the data collection. The half-time for equilibration is seen to decrease with increase in luciferase concentration as expected, from about 10 min for 20 μM to 3 min for 50 μM. In all measurements we have therefore allowed an appropriate equilibration time, 15–30 min, before taking data. The remaining results in table 3 (rows 9–26), are for solutions of lower ionic strength, viz., 0.05 M P_i. Row 9 refers to lumazine protein alone; five samples of different concentrations were measured and no concentration dependence was observed. The fluorescence intensity decay parameters are the same as those in table 1, including the decrease in τ_1 accompanying the protein complex formation, and therefore these data are not included here.

By comparing rows 4 and 18 we see that twice as much luciferase is required to achieve the same ϕ_{av} in 0.05 M P_i as in 0.25 M, confirming the ionic strength effect observed by Visser and Lee [2]. Dodecanol has been found to be an effective stabilizing agent for the bioluminescence activity of intermediate II [13]. Rows 10–17 in table 3 show that the addition of dodecanol to the luciferase-lumazine protein mixture increases ϕ_{av}, and this can be directly interpreted as a strengthening of the protein-protein interaction. For row 13 we can estimate $K = 38$ μM, about the same as in 0.25 M P_i. The changes in [E] and [LumP] are due to dilution as the dodecanol aliquots are added. The data are plotted in fig. 6 and show a clear break at a dodecanol concentration of 84 μM, twice that of luciferase. This suggests the possibility of two strong binding sites for dodecanol on the luciferase but a systematic titration study needs to be performed before this can be concluded with certainty. It is relevant to note, however, that it has been observed in this laboratory (M. Ahmad, unpublished data) that a 2:1 dodecanol/luciferase ratio is optimal for the stabilization of intermediate II. In contrast to the effect of dodecanol, the substrate for the bioluminescence reaction, tetradecanal, has no effect on the value of ϕ_{av} (rows 19–20). The same lack of effect was previously shown for decanal [2]. In

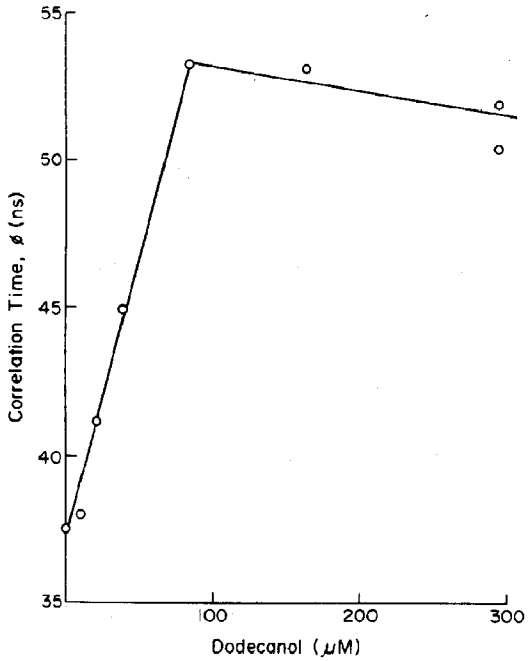

Fig. 6. The rotational correlation time of lumazine protein (about 30 μM) in the presence of *V. harveyi* luciferase (40 μM) is increased on the addition of dodecanol (2° C; pH 7.0, 0.05 M P_i).

row 21, it is seen that β,γ-dimyristoyl-L-α-lecithin also lengthens the ϕ_{av}, although not as much as with the same concentration of dodecanol. For lecithin, $K = 16$ μM.

Rows 22–26 in table 3 are for *P. phosphoreum* luciferase and lumazine protein. Again as previously observed, the association is much weaker than for *V. harveyi* luciferase; for row 22, $K = 220$ μM. With this preparation of higher specific activity than that used by Visser and Lee [2], the K value is correspondingly several times smaller. For this protein-protein equilibrium, the addition of dodecanol appears to be less effective for changing ϕ_{av} than it is for *V. harveyi* luciferase; for row 26, $K = 100$ μM.

4. Discussion

The goal of this work was first to establish that the luciferase had satisfactory binding homogeneity and, this being the case, we can now conclude with more confidence that the binding constant is too weak for a pre-equilibrium model to account for the bioluminescence property of lumazine protein. From the data of tables 1 and 2 and the binding plots, it is clear that the majority of the luciferase used here does complex with lumazine protein. Although the earlier study was made with luciferase purified from the same species of bacterium, *V. harveyi*, in the present work we have obtained the luciferase from a dark mutant in which the luciferase does not turn over because these bacteria do not manufacture the aldehyde substrate, and consequently do not generate bioluminescence. It is found that the luciferase from this mutant has several times higher activity for the in vitro bioluminescence reaction than does the wild-type luciferase. It is also observed that this luciferase from the mutant sustains a loss of activity after it having reacted to yield intermediate II, where no aldehyde substrate is present and light emission occurs. Table 1 shows that this reacted luciferase of lower activity still contains good binding competence, however, because the value of ϕ_{av} is about the same as that for luciferase of full activity (row 4).

The second conclusion that can be made concerns the question raised in previous work about the relationship between the protein-protein association constant and the interaction constant, K_i, for bioluminescence spectral shifting. The K_i is obtained from a reciprocal plot of the bioluminescence shift vs. lumazine protein concentration and is just that concentration of lumazine protein required to produce half the total spectral shift. A pre-equilibrium model requires that K_i be the same or greater than the equilibrium constant but the reverse is the case. For all preparations of *V. harveyi* luciferase including that from the wild-type previously studied, the equilibrium constant is several times higher than the K_i. For the present preparation of *P. phosphoreum* luciferase which has a higher bioluminescence activity than before, the association with lumazine protein is tighter but its equilibrium constant is still more than an order of magnitude larger than K_i. Additionally, the equilibrium is attained slowly. From the equilibration study in table 3 and the equilibrium constant for this preparation of 50 μM (0.25 M

P_i), the rate constants can be approximated to be about 150 M^{-1} s^{-1} for association and 2×10^{-3} s^{-1} for dissociation. Long equilibration times were not used in the spectral shifting study of Lee [3]. Therefore, in the bioluminescence interaction study, equilibrium was not established and K_i must have reflected more a kinetic competition between two reaction pathways, eqs. 19 and 20:

$$E + FMNH_2 + O_2 \rightarrow II \quad (16)$$

$$II + RCHO \rightarrow FT \quad (17)$$

or

$$II + RCHO \rightarrow FT^* \quad (18)$$

$$II + RCHO + FT \rightarrow FT^* + P \quad (19)$$

$$II + RCHO + LumP \rightarrow LumP^* + P \quad (20)$$

Luciferase reacts with $FMNH_2$ and O_2 to form the intermediate II (eq. 16), which then reacts with aldehyde to form a fluorescent transient (FT) species (eq. 17). This FT is presumably the in vitro bioluminescence emitter because it has a fluorescence spectrum indistinguishable from the bioluminescence and the kinetics of the fluorescence intensity are strongly linked to the decay of bioluminescence intensity [14]. Kurfuerst et al. [15,16] proposed eq. 18, where the excited state, FT*, is formed directly in the chemical reaction. From their detailed kinetic study, Matheson and Lee [14] concluded rather that the major pathway to light emission was by eq. 19, a sensitized, i.e., indirect, bioluminescence, where the reaction energy is deposited onto an FT molecule already accumulated in the system. Thus, the FT present and LumP when added would compete for the reaction exergonicity resulting in a bioluminescence emission that is a mixture of their fluorescence spectra.

Both the spectral shift and the K_i derived from it will thus depend on this competitive situation. For *P. phosphoreum* luciferase the FT is less stable than for *V. harveyi* luciferase (unpublished results) and it is correspondingly observed that LumP is more effective at shifting the bioluminescence from *P. phosphoreum* luciferase than for the reaction using the *V. harveyi* enzyme [3]. Also, it is observed in the reaction with *V. harveyi* luciferase that, if FT is allowed to accumulate before adding lumazine protein, the K_i becomes much larger [22].

We found excellent agreement in repeat measurements of the correlation time of a protein sample like lumazine protein and quite satisfactory sample-to-sample variation (e.g., see fig. 2). This gave us confidence that rather accurate measurement of the protein association system was attainable. In the mixture with luciferase, however, an increase in variability was found, in spite of careful attention to controlling the condition of measurement. On top of this there is a luciferase preparation variability resulting in differing binding efficacy. This variability may be a manifestation of the observation made earlier by Visser and Lee [2] who noted a lack of full binding competence of their luciferase for lumazine protein. It is conceivable that the luciferase purified from the mutant, MAVA, as distinct from the wild-type, has minor modifications in surface charge groups, which influence its interaction with lumazine protein with no effect on the in vitro bioluminescence activity with $FMNH_2$ and tetradecanal. The luciferase from different preparations might also be changed in this way, as the preparations were from different fermentation growths, harvested probably at different times in the growth cycle, and certainly started from different clones of the bacterium.

The phosphate and dodecanol effects, strengthening the binding of lumazine protein to luciferase and stabilizing the intermediate II, probably have a common origin with the preparation variability of binding discussed above, also arising from changes in the properties of surface groups. From correlation time results it has been proposed that the luciferase experiences a large conformational change when forming intermediate II [10]. By a hydrophobic interaction, the binding of dodecanol may favor this conformational change, a change also involved in the lumazine protein interaction (see below). The titration data in fig. 6 are very suggestive of two binding sites for dodecanol being involved, in interesting parallel to the optimal concentration of dodecanol for the stabilization of intermediate II. In this regard, the effect of phosphate is indirect: it affects protein hydration and

in that manner influences the hydrophobic interaction.

Since the phospholipid lecithin also strengthens the lumazine protein-luciferase association, it appears that this hydrophobic effect is nonspecific in nature. With *P. phosphoreum* luciferase, dodecanol strengthens the association by about the same factor as for the *V. harveyi* enzyme, although the association is weaker to begin with. It has been observed that in extracts of *P. phosphoreum*, the lumazine protein and luciferase are much more tightly associated than are the purified proteins [17]. It can be suggested then that in vivo there is a 'glue' that is some lipid which has been purified away in making homogeneous protein and this idea could be confirmed by testing lipid extracts from *P. phosphoreum* for their effect on the protein association.

Both luciferase and lumazine protein are roughly spherical proteins with axial ratios of less than 1.5 and 1.2, respectively [18,19]. Their measured ϕ values, therefore, are in reasonable agreement with eq. 7. The calculated value for the rotational correlation time of the E · LumP complex, assuming a spherical shape and the same hydration as luciferase (0.35 g/g), is about 80 ns at 2° C. That is why Visser and Lee [2] estimated the value at 75 ns. The results of table 2 and fig. 3 (upper line) are in good agreement with this but the other luciferase preparation gives 123 ns for the complex, 50% higher than calculated for a sphere. Since its binding curve in fig. 4 does not suggest aggregation to a higher molecular weight complex than 1 : 1, a possible explanation is that this complex is nonspherical.

For ellipsoids of revolution the rotational correlation time derived from anisotropy decay will depend on both the axial ratio and the angle between the transition dipole and symmetry axis of the ellipsoid (see ref. 20 and references cited therein). In general, the anisotropy decay function can be quite complex with up to five exponential terms expected. In one case, where the transition dipole of the fluorophore lies parallel to the symmetry axis of a prolate ellipsoid, the anisotropy would decay as a single exponential, with a correlation time increased over that expected for a sphere by a factor coincidently about the same as the axial ratio. A useful graphical presentation of the calculated correlation time dependence on axial ratio and angle with the transition dipole has been presented by Berkout et al. [21].

In most measurements of anisotropy decay and certainly in the present experiments, an anisotropy decay more complicated than single exponential cannot be recovered from the data due to the statistics. For the purpose of analysis, in fact it had to be assumed that the complex had a single rotational correlation time, depending on the luciferase, viz., 74 or 123 ns. To account for this latter value, a minimum axial ratio of 2 would be required of the complex [21]. It is relevant to note that the intermediate II also shows a high value of ϕ, interpreted also as resulting from a conformation of high axial ratio [10].

The application of fluorescence dynamics to the study of bioluminescent proteins has the advantage that these proteins naturally contain highly fluorescent ligands. The present results show that significant new information can be recovered about stoichiometry and the equilibrium parameters. In addition, there has been a bonus of new information about the dodecanol-binding sites and the effect of lipid on the association. Although there remain unknown factors which affect the equilibrium parameters and the shape of the complex, these have not been previously recognized nor is this information easily available from other techniques.

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