Association between Lumazine Protein and Bacterial Luciferase: Direct Demonstration from the Decay of the Lumazine Emission Anisotropy†

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ABSTRACT: The correlation time for the decay of the emission anisotropy of the bound 6,7-dimethyl-8-ribityllumazine in the lumazine protein from *Photobacterium phosphoreum* is 23 ns (3 °C). In the presence of about 100 μM concentrations of the bacterial luciferase purified from *Vibrio harveyi*, in about 10 times molar excess over the lumazine protein, the correlation time is increased to around 75 ns, and the anisotropy decays as a single exponential. This is a direct demonstration of association between luciferase and lumazine protein, and this is confirmed by sedimentation velocity measurements. The sedimentation coefficient for lumazine protein alone, $s_{20,w}$ = 1.9 S, is increased to 5.6 S in the luciferase solution. At lower luciferase concentrations the anisotropy decay becomes biexponential, and a constrained correlation times analysis is used to extract amplitudes of the shorter and longer times as measures of the free and complexed lumazine protein concentrations. With the use of this technique for analysis, a titration with lumazine protein shows that luciferases, which have high activities in the regular in vitro assay, behave nonstoichiometrically in respect to interaction with lumazine protein. For one preparation of *V. harveyi* luciferase, only 43% of the total protein showed a "competence" for binding lumazine protein. A Scatchard analysis was consistent with a 1:1 stoichiometry between lumazine protein and competent luciferase in the complex, with a value of K_d = 2–3 μM. In the presence of a high concentration (>100 μM) of *P. phosphoreum* luciferase, the emission anisotropy decay of lumazine protein again becomes biexponential, with a longer correlation time of 70 ns, indicative of complex formation. In this case the binding is much weaker, K_d < 900 μM (3 °C) in terms of total luciferase concentration. Addition of decanal or dodecanal has no effect on the binding for either luciferase, but for *V. harveyi* luciferase an increase of phosphate concentration dramatically decreases K_d. With the luciferase from *V. fischeri* no protein–protein association with lumazine protein is observed. No binding is found for the free 6,7-dimethyl-8-ribityllumazine to luciferase.

Bioluminescent bacteria of the genus *Photobacterium* produce lumazine protein in high yield (Gast & Lee, 1978; Lee & Koka, 1978; Lee & Elrod, 1981). This soluble protein of 20 000 daltons is called lumazine protein because it contains 6,7-dimethyl-8-ribityllumazine (Koka & Lee, 1979) (hereinafter called "lumazine") as a bound prosthetic group (K_d = 0.05 μM, 2 °C; Visser & Lee, 1980; Irwin et al., 1980). The bound lumazine is efficiently fluorescent (Q_F[1] = 0.6, τ_F = 14.4 ns, 3 °C; Visser & Lee, 1980; Koka & Lee, 1981) and has a fluorescence maximum, 475 nm, and spectral distribution identical with that of the bioluminescence from these cells (Gast & Lee, 1978; Lee et al., 1981; Lee & Elrod, 1981).

It has been proposed that the function of lumazine protein in the cells is to radiate the bioluminescence energy. It appears to act as an acceptor in an energy-transfer or dye-sensitized chemiluminescence reaction (Gast & Lee, 1978; Matheson et al., 1981; Lee et al., 1981). This proposal is supported by the fact that, if lumazine protein is added in sufficient quantity to the in vitro bioluminescence reaction, the bioluminescence is emitted in a spectral distribution identical with that of the fluorescence of lumazine protein (Gast & Lee, 1978; Lee et al., 1981; Lee & Elrod, 1981). This reaction uses a soluble enzyme bacterial luciferase (M_r 80 000) and the substrates $FMNH_2$, oxygen, and dodecanal or other aliphatic aldehydes.

It has also been proposed that the chemical reaction on luciferase produces a primary electronic excited species, of energy equivalent to a fluorescence maximum around 400 nm (Matheson et al., 1981; Lee et al., 1981). Under certain reaction conditions an in vitro bioluminescence with a maximum at 390 nm has in fact been reported (Matheson et al., 1981). The primary species needs to have an energy of at least this amount in order to pump the lowest electronic transition of lumazine protein, which has an absorption band with a single maximum in the visible at 416 nm (Small et al., 1980; Visser & Lee, 1980; Koka & Lee, 1981). In the absence of lumazine protein other fluorescent species present in the reaction mixture may assume this energy-acceptor function (Matheson et al., 1981; Matheson & Lee, 1981).

Efficient bioluminescence should be favored if the reaction takes place in a preformed complex of the luciferase and lumazine protein. Depending on the excited-state lifetime of the primary species, energy transfer to the lumazine protein in such a complex would not have to depend on the encounter rate between the two proteins, and competition from other routes of decomposition would be minimized. Some preliminary reports of such a complexation have previously been made (Gast et al., 1978; Koka & Lee, 1981; Lee et al., 1981).

The lumazine group is an excellent intrinsic luminescence probe by virtue of its efficient blue fluorescence, its strong and rigid binding to the lumazine apoprotein, and the value of its fluorescence lifetime (Visser & Lee, 1980; Lee et al., 1981; Koka & Lee, 1981). In forming a complex with luciferase, the rotator to which the lumazine is bound will change in molecular weight, from 20 000 to 100 000. The long fluorescence lifetime and high fluorescence efficiency of the bound lumazine are well suited to using the relaxation rate

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[1] Abbreviations: Q_F, fluorescence quantum yield; τ_F, fluorescence lifetime; ϕ, rotational correlation time; FMN, flavin mononucleotide; *A*, absorbance; P_i, inorganic phosphate; S, Svedberg unit, 10^{-13} s; LumP, lumazine protein; $FMNH_2$, reduced flavin mononucleotide; DEAE, diethylaminoethyl; BSA, bovine serum albumin; NMR, nuclear magnetic resonance.

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of the lumazine emission anisotropy to measure the protein–protein association.

When lumazine protein is excited in its visible absorption band by a short pulse of polarized light, the anisotropy (r) of the fluorescence, viewed at 90° to the direction of the exciting beam, is found to change in a manner described by a single exponential function (Visser & Lee, 1980): $r(t) = r_0 \exp(-t/\phi)$, where r_0 is the initial anisotropy, ϕ is the rotational correlation time, and $r = 2p/(3 - p)$, where p is the polarization. This result implies that lumazine protein is a spherical rotor and that the lumazine is inflexible during the fluorescence lifetime. In this case ϕ is related to the molecular weight, M_r, partial specific volume, $\bar{v}$, viscosity of the solution, η, temperature, T, and degree of hydration, h, of the protein by $\phi = M_r\eta(\bar{v} + h)/(RT)$. For lumazine protein, $\phi = 21.7$ ns (3 °C), and given the value of M_r 20 000 (from other methods), the degree of hydration $h = 0.8$ cm^3 g^{-1}.

If lumazine protein and luciferase form a complex with M_r 100 000, then ϕ should undergo a significant increase, calculated around 100 ns (3 °C) if the complex retains a spherical shape and same relative degree of hydration. In an equilibrium mixture of the two proteins, conditions might be such that some lumazine protein is bound to luciferase and some is free. The total emission anisotropy should then exhibit a two-exponential decay:

$$r(t)/r_0 = \alpha_1 \exp(-t/\phi_1) + \alpha_2 \exp(-t/\phi_2)$$

with ϕ_1 and ϕ_2 the rotational correlation times of free and bound lumazine protein, respectively, and the constants α_1 and α_2 measures of their amounts, provided that free and bound lumazine protein have the same fluorescence quantum efficiency. In this paper we report the unambiguous observation of the protein–protein association and use the analysis of the anisotropy decay function to describe the equilibrium.

Materials and Methods

Materials. Lumazine protein was purified from *P. phosphoreum* strain A13 by the method previously described by Small et al. (1980). The preparation, purified through the DEAE-Sepharose stage, showed no flavoprotein contamination. Its molar concentration was calculated from the visible absorbance, since the extinction coefficient of free lumazine (ϵ_{416} = 10 300 M^{-1} cm^{-1}) is hardly changed on binding (Koka & Lee, 1981), and the stoichiometry of binding is consistent with 1:1 (Small et al., 1980; Visser & Lee, 1980; Koka & Lee, 1981).

The luciferases used were purified from the bacteria *Vibrio* (formerly *Beneckea*) *harveyi*, strain 392, *Vibrio* (formerly *Photobacterium*) *fischeri*, strain 399 (Reichelt & Baumann, 1973; Baumann et al., 1980), and from *P. phosphoreum*, strain A13 (Fitzgerald, 1978). Procedures for extraction and purification to protein homogeneity were modifications of those previously used for *V. harveyi* and *V. fischeri* (Lee & Murphy, 1975) and for *P. phosphoreum* by Gast et al. (1978) and are to be described elsewhere. The important modification was the employment of a constant ionic strength elution of the luciferases alternately from DEAE-Sephadex A-50 and DEAE-Sepharose, until the content of accompanying fluorophores was reduced to a tolerable level. One index of purity of the luciferase preparation was the absorbance ratio A_{280}/A_{450}. By acid precipitation it could be shown that the A_{450} was quantitatively attributable to a flavoprotein, although flavin was not the sole contributor to the background fluorescence. The maximum values of the A_{280}/A_{450} ratios for preparations used in this present work were as follows: *P. phosphoreum*, 150; *V. fischeri*, 200; *V. harveyi*, >600. These ratios correspond to an approximate molar percent presence of a flavin in the luciferase of 5, 4, and <1, respectively.

The concentrations of luciferases were determined from published extinction coefficients (280 nm) and an average value of M_r 80 000 (Gunsalus-Miguel et al., 1972; Yoshida & Nakamura, 1973). Bioluminescence activity was determined in two ways with photoreduced $FMNH_2$ (Lee, 1972; Lee & Murphy, 1975). The first was the maximum initial light intensity assay under optimal conditions (21 °C) as described before (Lee, 1972). The other was to measure the specific integrated light yields under optimized conditions with tetradecanal: *V. harveyi*, 1.5; *V. fischeri*, 4.2; *P. phosphoreum*, 5.0; units are 10^{14} photons A_{280}^{-1}. Absolute light standardization was by reference to the NBS standard lamp via the luminol chemiluminescence reactions (Lee et al., 1966).

Authentic 6,7-dimethyl-8-ribityllumazine was the generous gift of Professor Dr. Wolfgang Pfleiderer, University of Konstanz, West Germany, and FMN (Fluka, Buchs, Switzerland) was purified to about 90% by DEAE chromatography. All other chemicals were of the best commercial grades. The standard buffer used for all experiments was 50 mM P_i with 5 mM 2-mercaptoethanol, pH 7.

Fluorescence Measurements. Steady-state fluorescence polarization was determined as in Visser & Lee (1980), with the same conditions for excitation and emission and allowing for background contribution from the fluorescence of the luciferase preparation where necessary. Fluorescence decay lifetime and relaxation of emission anisotropy were determined by exciting with picosecond pulses from a mode-locked Ar_+ laser (458 nm). The instrumentation and data analysis are described in detail elsewhere (Visser & Müller, 1980; Visser & van Hoek, 1979; Visser & Lee, 1980; Visser et al., 1980). We used a nonlinear least-squares method to analyze the data (Grinvald & Steinberg, 1974). The parameters (α_i; ϕ_i) for the best fit of $r(t)$ were obtained after a search for the minimum value of χ^2:

$$\chi^2 = 1/(N - f)\sum_{i=1}^{N} W_i[r(i) - r_c(i)]^2$$

in which the summation is made over N channels, f is the number of adjustable parameters, $r(i)$ is the experimental anisotropy in channel i, $r_c(i)$ is the calculated one, and W_i is a weighting factor. For calculation of W_i an analytical expression derived by Wahl (1979) was used. By integrating $r(t)$ over time we can also obtain a value of the steady-state anisotropy ($\langle r \rangle$).

Ultracentrifugation. A Beckman Model E ultracentrifuge fitted with absorption optics was used for these studies. Both double-sector cells of 12-mm path length or a single-sector 1.2 mm path length cell were used, depending on the total protein concentration. Luciferase alone was usually present in the reference cell. Sedimentation equilibrium measurements were made at 6000 rpm, and sedimentation velocity measurements were made at 60 000 rpm. For the equilibrium case, M_r was calculated from the average slope of the ln c vs. r^2 plot, taken across the entire tube length. The concentration of lumazine protein was followed by its absorbance at 414 nm.

Results

Anisotropy Decay. Since most of the results to be presented here are retrieved from time-dependent anisotropy measurements, we will first show the constituent curves together to demonstrate the method of procedure. Figure 1 is the result for a typical experiment on lumazine protein in the presence of bacterial luciferase. The impulse-response function $g(t)$ is nearly a δ function so that its deconvolution from the

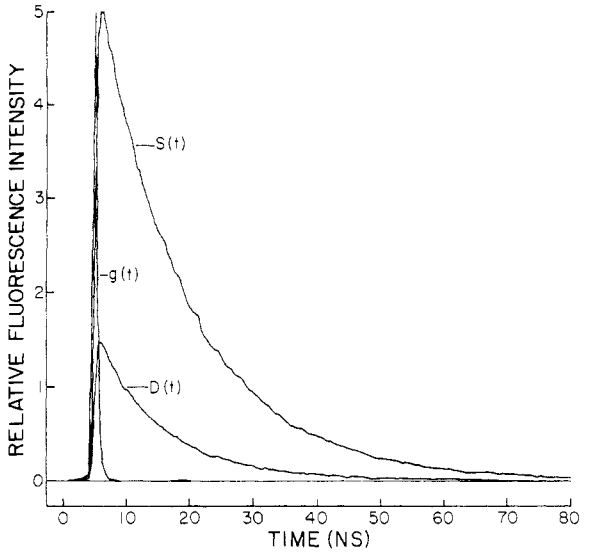

FIGURE 1: Lumazine protein in the presence of bacterial luciferase (2 °C). Instrumental response to the laser pulse is $g(t)$, the difference curve for the polarized fluorescence is $D(t) = I_{\parallel}(t) - I_{\perp}(t)$, and the total fluorescence is $S(t) = I_{\parallel}(t) + 2I_{\perp}(t)$.

fluorescence decay is unnecessary. The difference curve $[D(t) = I_{\parallel}(t) - I_{\perp}(t)]$ has been scaled to the normalized sum curve $[S(t) = I_{\parallel}(t) + 2I_{\perp}(t)]$, representing the total fluorescence, where $I_{\parallel}$ and $I_{\perp}$ are the polarized fluorescence intensities. The maximum of the $g(t)$ function is taken for the starting point of the analysis. The maximum channel of the total fluorescence usually contained about 150000 counts. The involvement of rotational motion at a rate comparable to that of the fluorescence decay causes a noticeable difference in time evolution of the two functions. The time span to obtain an anisotropy decay curve, $r(t) = D(t)/S(t)$, of good quality is limited by the fluorescence lifetime of the fluorophore. With the fluorescence lifetime of lumazine protein around 14 ns, a time span of about 70 ns can be employed. The signal to noise ratio of the anisotropy increases with time as the fluorescence intensity diminishes. Moreover, since $D(t)$ and $S(t)$ are constructed from the individual polarized intensities, noise propagates into $r(t)$ as well. It was therefore essential to find a good compromise between a long time for data collection and the long-term stability of the apparatus. For comparative purposes only the plots of $r(t)/r_0$ and the resulting fits are presented.

Figure 2 shows that the correlation time, ϕ, of lumazine protein, derived from the decay of its emission anisotropy, is increased in the presence of luciferase. In the top panel of Figure 2 the steeper line is for lumazine protein alone (11 μM), and its relative anisotropy $[r(t)/r_0]$ decays as a single exponential with $\phi = 23$ ns (2 °C). A comparable result has been published earlier under slightly different experimental conditions (Visser & Lee, 1980). On the addition of a large molar excess of *V. harveyi* luciferase (123 μM; top panel), the lumazine's anisotropy decay remains a single exponential but with ϕ increased to 75 ns. This is a direct demonstration of protein–protein association between lumazine protein (LumP) and the luciferase (E): E + LumP $\rightleftharpoons$ E–LumP.

The middle panel of Figure 2 is for lumazine protein and an excess of *P. phosphoreum* luciferase. The data have been fitted to a double exponential, $\alpha_1 = 0.15$, $\phi_1 = 22$ ns, $\alpha_2 = 0.14$, and $\phi_2 = 61$ ns. Evidently under these conditions there is a short component attributable to free lumazine protein and a long component from the heavier complex, both contributing to the overall anisotropy decay.

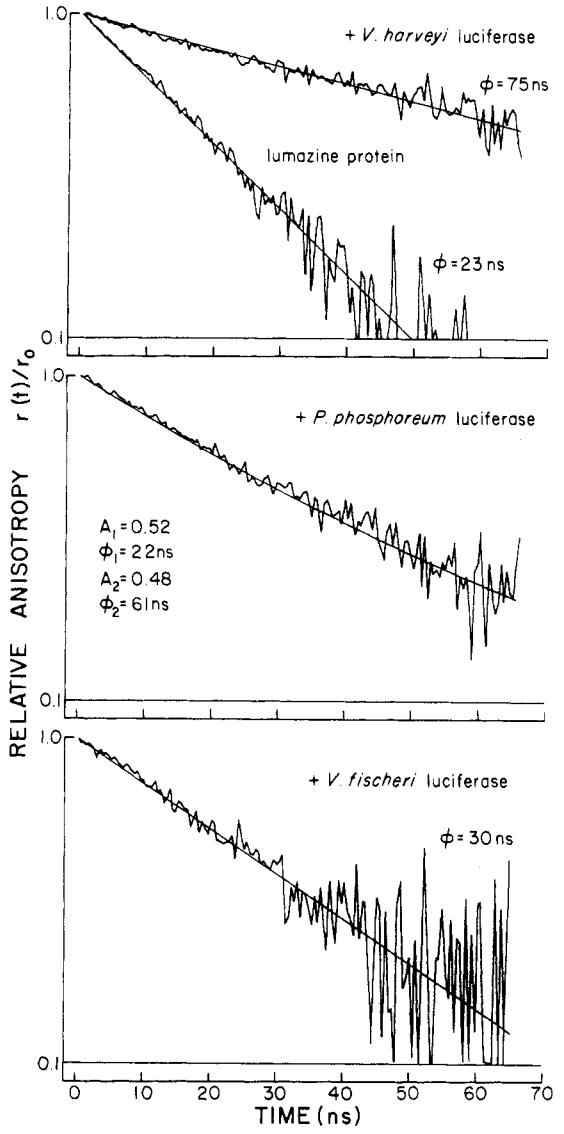

FIGURE 2: Plots of relative emission anisotropy for free or complexed lumazine protein. All at 2 °C in 50 mM P_i buffer, pH 7. The lines drawn through the noisy signals are the minimized χ^2 fits, all parameters free. (Top panel) Lumazine protein (11 μM) by itself (ϕ = 23 ns) and in the presence of *V. harveyi* luciferase (123 μM protein; ϕ = 75 ns). (Middle panel) Lumazine protein (36 μM) in the presence of *P. phosphoreum* luciferase (609 μM). (Lower panel) Lumazine protein (5.4 μM) and *V. fischeri* luciferase (900 μM). In this case the line is the fit to the decay in the presence of the same milligram concentration of BSA. Note that in this figure, as well as Figures 3 and 4, the ordinate scale is logarithmic.

The bottom panel of Figure 2 is for lumazine protein in the presence of *V. fischeri* luciferase. The anisotropy decays with a precise single exponential, $\phi = 30$ ns. The data are noisy because of the large amount of background that comes along with this luciferase preparation, and this has been subtracted from the original data points. A control measurement of lumazine protein in the presence of the same milligram concentration of BSA in place of luciferase gives the same result, $\phi = 30$ ns (the data are not shown, but the line is a fit to the BSA data). The lengthening of the rotational correlation time of the lumazine protein, therefore, in this case, can be accounted for entirely by an increase in the microviscosity due to the high protein concentration. There is no evidence for a protein–protein complex with *V. fischeri* luciferase.

Consistent with the above two-state equilibrium model, changes in temperature and concentration alter the relative contributions of the fast and slow rates to the overall anisotropy

Table I: Effect of Dilution on the Decay of the Emission Anisotropy of Lumazine Protein in the Presence of *V. harveyi* Luciferase[a]

lumazine protein (μM)	luciferase total protein (μM)	fluorescence lifetime (ns)	all parameters free[b]					φ's fixed[c]			average anisotropy	initial anisotropy[f]
			A_1[d] (%)	ϕ_1 (ns)	A_2[e] (%)	ϕ_2 (ns)	χ^2	A_1 (%)	A_2 (%)	χ^2	$\langle r \rangle$	r_0
12.8	400	13.5	15	8.7	85	83	1.0	13	87[g]	1.7	0.225	0.275
38	400	14.0	13	8.9	87	72	1.1	21	79[g]	1.4	0.219	0.277
19	200	14.0	18	13	72	69	0.92	17	83	1.3	0.239	0.303
6.4	66.7	14.2	40	14.8	60	64	0.90	34	66	3.1	0.161	0.286
3.2	33.3	14.2	79	23	21	107	1.03	67	33	1.2	0.191	0.272
1.6	16.7	14.4	100	23.4			1.4	71	29	1.25	0.165	0.263

[a] 2 °C, 50 mM P_i and 5 mM 2-mercaptoethanol, pH 7.0. [b] Two-exponential fit to equation $r(t)/r_0 = \alpha_1 \exp(-t/\phi_1) + \alpha_2 \exp(-t/\phi_2)$, allowing all parameters to vary to minimize χ^2. [c] The fit is made with the constraint $\phi_1 = 23$ ns and $\phi_2 = 60$ ns, except as noted. [d] $A_1 = 100\alpha_1/(\alpha_1 + \alpha_2)$. [e] $A_2 = 100\alpha_2/(\alpha_1 + \alpha_2)$. [f] $r_0 = \alpha_1 + \alpha_2$. [g] $\phi_2 = 75$ ns.

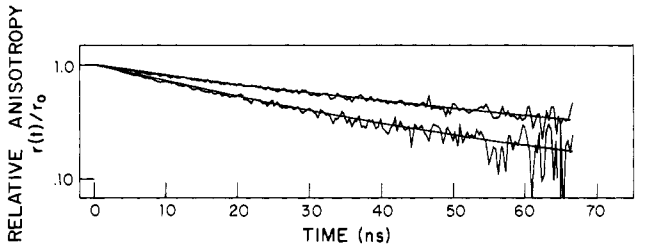

FIGURE 3: Effect of dilution on the bound lumazine's anisotropy decay; 3 °C, 50 mM P_i, and pH 7. The upper curve is for lumazine protein (19 μM) and *V. harveyi* luciferase (200 μM), and the steeper curve is for 3.2 and 33 μM, respectively.

decay. Figure 3 is the anisotropy decay for lumazine protein in the presence of about 10 times excess of *V. harveyi* luciferase. The upper curve in Figure 3 is for a total protein concentration of 220 μM, and the lower one is about a 6 times dilution of this. It is seen that for the higher concentration (upper curve) the longer correlation time predominates; the lumazine protein is mostly bound. On dilution (lower curve) the shorter lifetime predominates; more of the lumazine protein is free.

In Figure 3 the data are fitted to a two-exponential decay function, allowing all four parameters to assume values to minimize χ^2. In order to provide an internally consistent set of parameters, that is, ones that sensibly reflect the effects on a two-state equilibrium model that might be expected of the external changes, we have analyzed the data under the constraint that the values of ϕ_1 and ϕ_2 should be that of the free and bound lumazine protein, respectively. Therefore ϕ_1 should be set at 23 ns, and ϕ_2 should be in the range of 60–80 ns, depending on conditions like protein concentration.

Figure 4 shows a typical set of data analyzed by the two different methods. The upper panel is the fit obtained when all four parameters are allowed to find values that minimize χ^2. The lower panel is the constrained fit where the ϕ's are fixed. The constrained analysis results in $\chi^2 = 1.67$, increased only by a small amount over the unconstrained case $\chi^2 = 1.37$, and this, together with a comparison of the deviations in the topmost and lowermost panels, gives assurance that the two methods of analysis result essentially in equally good fits to the data. There is, however, a drastic difference in A_1/A_2, representing the ratios of the contributions of unbound protein and complex, for the two methods. Due to noise in the data and the limited time span, there is a rather large choice of values that give equally good fits. Generally, the constrained two-exponential fit leads to better results than a single exponential fit except in the case where luciferase is present in large excess.

In Table I the results of analyses by both unconstrained, all parameters free, and constrained, ϕ's fixed, methods are

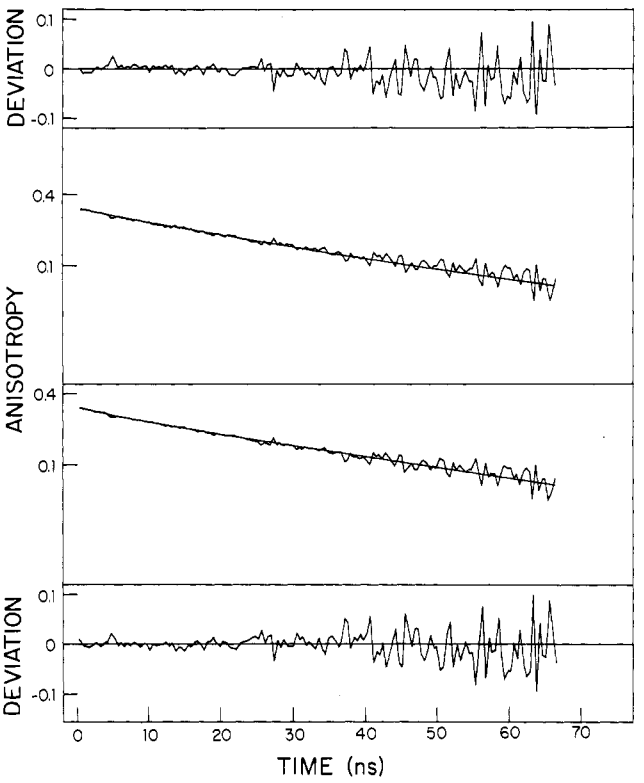

FIGURE 4: Anisotropy decay of lumazine protein (6.4 μM) in the presence of *V. harveyi* luciferase (14 μM); 2 °C, pH 7, and 50 mM P_i. (Upper panel) Unconstrained fit to a two-exponential decay: $A_1 = 20$, $\phi_1 = 6.4$ ns, $A_2 = 80$, $\phi_2 = 48$ ns, and $\chi^2 = 1.37$. (Lower panel) The fit is constrained to $\phi_1 = 23$ ns and $\phi_2 = 65$ ns and results in $A_1 = 48$, $A_2 = 52$ and $\chi^2 = 1.67$. Note the ordinate scale for the deviation is linear.

given. As can be seen, unconstrained analysis yields better results in terms of minimum χ^2 values, but the parameter values are highly uncorrelated; they can adopt a wide variety of values. For the constrained analysis, however, there is a more regular trend in the values of the amplitudes, A_1 increasing with dilution as expected. At the highest luciferase concentration used the lumazine protein is about 80% complexed and at the lowest concentration (last line) about 30%. At the highest luciferase concentration, 400 μM, we have set ϕ_2 a little higher to 75 ns, as an allowance for the contribution to the microviscosity from the protein. However, setting ϕ_1 a little higher too or fixing ϕ_2 at either 60 or 75 ns hardly makes any difference to the resulting values of the A's (Tables I and II). This is probably due to the limited usable time span of the experiments.

The change in concentration of free and bound lumazine protein is also reflected in the average anisotropy, $\langle r \rangle$, which is related to fluorescence polarization, p, by $\langle r \rangle^{-1} = (3/2)$-

Table II: Binding of Lumazine Protein to *V. harveyi* Luciferase: Effect of Decanal and Phosphate Concentration[a]

lumazine protein (μM)	luciferase total protein (μM)	A_1 (%)	ϕ_1 (ns)	A_2 (%)	ϕ_2 (ns)	χ^2	K_d[b] (μM)	remarks
15	15	76	23	24	70	1.07	9.0	3 °C
15	15	70	23	30	60	1.09	4.6	3 °C
15	15	69	23	31	60	1.2	4.0	3 °C, 5 mM decanal
15	15	87	13	13	45	1.2	30	22 °C
15	15	68	13	32	45	1.2	3.5	22 °C, 0.2 M P_i
30	15	48	13	52	46	1.15	4.7	22 °C, 0.2 M P_i
30	15	23	20	77	70	2.1	0.7	22 °C, 1 M P_i
30	15	13	20	87	70	1.2	<0.5	22 °C, 1 M P_i

[a] The emission anisotropy is fitted to a two-exponential decay with ϕ's constrained to the values shown. All at pH 7 with 2 mM 2-mercaptoethanol. [b] Calculated assuming 43% competent luciferase.

$[(1/p) - (1/3)]$; for free lumazine protein, $p = 0.23$ (Visser & Lee, 1980). Then $\langle r \rangle = 0.17$, to be compared with 0.165 on the last line of Table I. For the mostly bound case, $\langle r \rangle = 0.25$ and the calculated $p = 0.31$ is in rough agreement with the steady-state observation (see later). The necessity of subtracting a large contribution from the contaminating fluorophores in the luciferase detracts from the reliability of steady-state methods. There appears to be little change in the value of the initial anisotropy, r_0, with protein concentration.

It is also to be noticed in Table I that the fluorescence lifetime of lumazine protein in the complex, 13.5 ns, is slightly shorter than when it is unbound, 14.4 ns. The free lumazine molecule has a fluorescence lifetime of 10.0 ns (4 °C; Visser & Lee, 1980), and although a weaker binding of the lumazine in the complex could explain the decrease in lifetime, this is not supported by the following observations. In the complex there is no evidence for a second shorter fluorescence lifetime; the steady-state polarization is consistent with the lumazine being entirely bound to a 100 000-dalton species (see below), and the visible absorption spectra of free and complexed lumazine protein are identical, with a maximum at 416 nm. The free lumazine absorption maximum is 407 nm, and free lumazine does not bind to luciferase (vide infra).

The observations presented so far are consistent with the simple equilibrium model, but so that we can proceed further, certain assumptions need to be made explicit. The first is that A_1 and A_2 are proportional to the amounts of free and complexed lumazine protein, respectively. This is reasonable since the fluorescent properties (spectra; lifetimes) of free and complexed lumazine protein are nearly identical. The second is that we can determine the absolute concentrations of the two proteins participating in the equilibrium. For lumazine protein this is relatively unambiguous. Since the lumazine is bound to protein in a 1:1 stoichiometry (Small et al., 1980; Visser & Lee, 1980) and its extinction coefficient (bound) is 10 300 M^{-1} cm^{-1} (416 nm) (Koka & Lee, 1981), the lumazine protein concentration is determined from the visible absorbance. For the luciferase, however, we are only able to determine a total protein concentration. The following experiments will demonstrate that luciferase's ability to bind lumazine protein is far less than stoichiometric.

Figure 5 displays titration curves where the ratio of bound to free of the limiting component, given by the percentage $A_2 = 100\alpha_2/(\alpha_1 + \alpha_2)$, is plotted against increasing concentrations of the other component. The top panel is for 10 μM lumazine protein, and although a considerable excess of luciferase has to be added, the fraction of complexed lumazine protein approaches 100%. For the above equilibrium reaction it may be simply shown that $A_2^{-1} = 1 + K_d E^{-1}$. On a Benesi–Hildebrand plot (not shown) the points from the top panel fall on a straight line and give a value of $K_d = 6.5$ μM, with

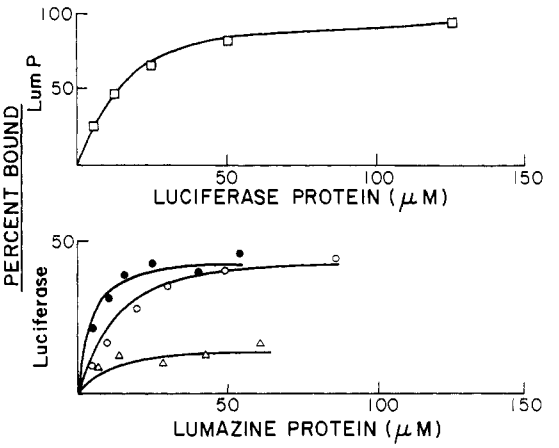

FIGURE 5: Titrations calculated from constrained two-exponential analysis of the bound lumazine's anisotropy decay (2 °C). (Top panel) Lumazine protein (10 μM) becomes completely complexed (long correlation time) as luciferase from *V. harveyi* is added in excess. (Lower panel) Two separate preparations, circles and triangles, of *V. harveyi* luciferase are used, with the lumazine protein added in excess. The percent luciferase bound saturates at less than 50%.

reference to luciferase total protein concentration. The curve in the top panel is reconstructed from this line of best fit.

The lower panel of Figure 5 is the reverse titration, where the luciferase is limiting. In spite of a considerable excess of lumazine protein most of the luciferase fails to complex. The measurements were made on two different batches of *V. harveyi* luciferase of the same specific activity. The open and closed circles are for one batch, at 62 and 18 μM protein concentrations, respectively. The triangles are for the other batch, at a concentration of 40 μM. The simple interpretation of these observations is that only a fraction of the total luciferase is competent for binding lumazine protein and the remainder not. From the saturation values in Figure 5, lower panel, we conclude that the first batch is 43% competent and the other 14%. Benesi–Hildebrand plots of these data using the total luciferase concentration are not linear but can be linearized by plotting against the concentration of competent luciferase, e.g., calculated as 43% of the total for the data represented by the circles. In this case $K_d = 3.0$ μM with reference to the competent luciferase concentration. The curves through the circles (open and closed) are reconstructed from the Benesi–Hildebrand lines of best fit. From the data in the top panel, we obtain $K_d = 2.8$ μM when the luciferase concentration is again set equal to 43% of the total luciferase protein concentration. The data represented by the triangles are too scattered for reliable analysis. The data in Table I were also obtained with the low-binding batch of luciferase, and 1 order of magnitude higher estimate of K_d results if the competence of the luciferase is not factored in.

Table III: Binding of Lumazine Protein to *P. phosphoreum* Luciferase[a]

lumazine protein (μM)	luciferase total protein (μM)	A_1 (%)	ϕ_1 (ns)	A_2 (%)	ϕ_2 (ns)	χ^2	K_d (μM)	remarks
35	50	100	20.8			1.9		$r_0 = 0.31$
35	115	100	22.1			1.1		
35	609	50	20	50	60	1.4	609	
34	1178	32	23	68	70	1.7	387	
50	30	100	18			1.5		$r_0 = 0.27$
50	65	100	18.6			1.4		
50	128	100	20			1.2		
50	241	78	18	22	70	2.9	843	
50	580	66	18	34	70	1.4	1102	
100	600	64	23	36	70	1.4	1066	
10	50	100	23			1.0		
10	50	100	25			1.5		150 μM dodecanal
36	609	61	23	39	70	1.4	953	
36	609	66	23	34	70	1.4	1182	150 μM dodecanal
36	609	55	23	45	70	1.1	744	600 μM dodecanal

[a] All at 3 °C; the two-exponential decays are analyzed with ϕ's constrained to the values shown. Where A_2 and ϕ_2 are not given, it means that the decay is accurately single exponential.

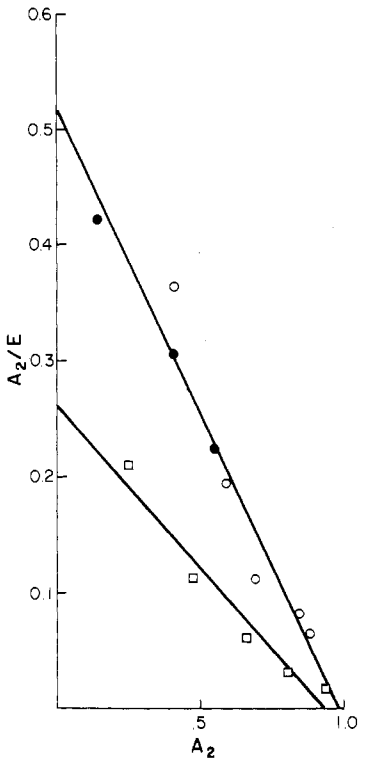

FIGURE 6: Scatchard analysis of the data in Figure 5 with corresponding symbols. A_2 is from the two-exponential analysis of the anisotropy and represents the fraction-bound species. Note that two of the filled points from Figure 5 are omitted since the 43% binding assumption leads to unacceptable values of A_2.

The data from Figure 5 (excluding the triangles) are subjected to a Scatchard analysis in Figure 6. Both sets of data show a satisfactory least-squares fit to a straight line, and the intercept of near unity on the x axis supports the 1:1 stoichiometry of the simple equilibrium model. The squares are the data from the top panel of Figure 5, and the K_d calculated from the y intercept is 3.9 μM. Corrected for the 43% competence, this becomes 1.7 μM. The other set of data is corrected with respect to competent luciferase concentration before plotting and yields a K_d = 2.0 μM.

Figure 7 is a van't Hoff plot; the slope gives $\Delta H°$ = 20 kcal mol^{-1} for the protein–protein association. The values chosen for the ϕ's in the constrained analysis were calculated from the measurements at 2 °C (Table I) with allowance for change in temperature and solution viscosity.

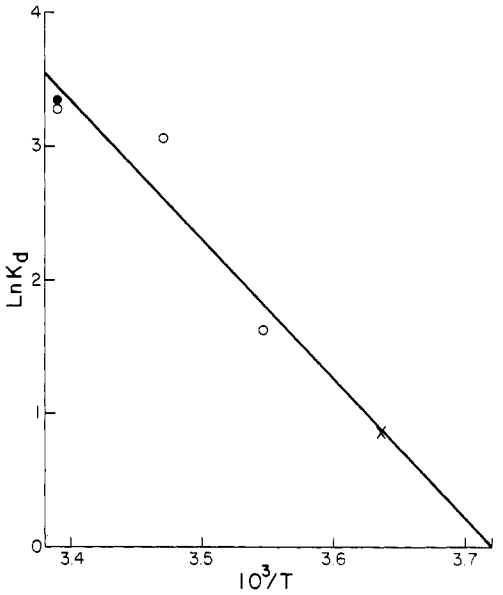

FIGURE 7: Temperature dependence of the lumazine protein–luciferase (*V. harveyi*) interaction. The cross point is the grand average value for 2 °C, K_d = 2.4 μM. (Open circles) Lumazine protein (15 μM) and competent luciferase (43% total protein, 15 μM). (Filled circle) Lumazine protein (11 μM) and competent luciferase (83 μM).

Table II shows that the addition of decanal has no significant influence on the binding of lumazine protein to *V. harveyi* luciferase. Decanal is one of the substrates in the bioluminescence reaction. In contrast, an increase in phosphate concentration has a dramatic effect. Even by 0.2 M P_i, the K_d at room temperature is decreased by almost 10-fold from that in 50 mM. By 1 M P_i the K_d is below 1 μM (at room temperature!). Again the values chosen for the ϕ's are adjusted for the increase in viscosity of the solution.

Observations demonstrating an interaction of lumazine protein and *P. phosphoreum* luciferase are in Table III. Titration behavior can be seen, as the luciferase concentration increases so does the contribution of the heavier species (A_2). However, it is evident that the binding of lumazine protein to *P. phosphoreum* luciferase on a total protein basis is much weaker than to *V. harveyi* luciferase. The average K_d in Table III is 900 μM (3 °C), and such a value makes analysis of the data rather difficult. If we fix luciferase at 600 μM, we cannot construct a titration curve like Figure 5 that looks meaningful.

Table IV: Steady-State Fluorescence Polarization[a]

luciferase (μM)		lumazine[b] (μM)	lumazine protein (μM)	average polarization[c] p
V. fischeri:	590	0		0.323
	590	13		0.028
V. harveyi:	590	0		0.267
	590	13		0.069
	590	25		0.067
	590	25		0.051[d]
	400		38.4	0.362
	17		1.6	0.27

[a] All at 2 °C in standard buffer except as noted. [b] 6,7-Dimethyl-8-ribityllumazine. [c] Luciferase background contribution is subtracted when lumazine or LumP is included. [d] Room temperature.

Even with 100 μM lumazine protein, only 36 μM or 6% of the total luciferase is in the complexed state. If this 6% represents the content of competent luciferase, then the real K_d reduces to 20–50 μM, a range still rather higher than that found with *V. harveyi* luciferase.

Table III also shows no effect of dodecanal addition on the equilibrium. *P. phosphoreum* luciferase has a higher bioluminescence activity with dodecanal than with decanal. At low luciferase concentrations there is no increase in binding due to dodecanal, and at high luciferase there is no change in the binding.

Steady-State Fluorescence Polarization. Direct measurement of the average polarization was used to establish that free lumazine does not interact with luciferase. Table IV shows that the contaminating fluorophores in the luciferase preparation produce a highly polarized fluorescence. *P. phosphoreum* luciferase is similar (not shown). This background fluorescence level contributes about 25% of the total intensity in the case of the experiment with 13 μM lumazine. When properly subtracted, the net polarization due to the lumazine is very small, consistent with no binding.

Mixing lumazine protein (38.4 μM), on the other hand, with luciferase (400 μM) gives a net polarization much higher than for lumazine protein alone. It is attained immediately, that is, within the several minutes required for the measurement, and is constant for at least 24 h (4 °C). On dilution of the mixture the polarization decreases toward the 0.23 value of lumazine protein itself (Visser & Lee, 1980). All these results are consistent with the protein–protein interaction suggested more directly by Figures 2 and 3.

Sedimentation Studies. Table V summarizes the results of the ultracentrifuge measurements. With a slight protein excess of *P. phosphoreum* luciferase, lumazine protein displays a molecular weight unchanged from free solution, 16 900 ± 900 [cf. Small et al. (1980)]. The ln c vs. r^2 plot is linear (not shown). There is no indication of any higher molecular weight species. At higher luciferase concentrations, Table V shows that the M_r of the bound lumazine increases to an average around 40 000. The presence of a high molecular weight luciferase-bound lumazine protein may be inferred from these data, but the ln c vs. r^2 plots are not so linear as the first case (they "bend" the wrong way in fact), the individual results are rather scattered, and if calculations are to be attempted with these data, rather doubtful assumptions have to be made concerning ideal behavior of solutions at these high protein concentrations, 20–40 mg/mL. A control experiment with BSA, 34 mg/mL, does not show any molecular weight enhancement of the bound lumazine; the apparent low result is spurious, probably a result of nonideality. The table also shows

Table V: Ultracentrifuge Study of Lumazine Protein-Luciferase Interaction[a]

luciferase total protein (μM)		lumazine protein (μM)	sedimentation equilibrium M_r	sedimentation coefficient $s_{20,w}$ (S)
P. phosphoreum:	59	42	17 000[b]	
	293	42	42 000	
	257	56	39 000	
	553	32	55 000	
	500	113	36 000	
	179	17[b]		2.2
V. harveyi:	440	122	38 000	
	130	0		4.6[d]
	130	20		5.6
BSA:	520	129	11 000[c]	

[a] All at 2 °C in standard buffer with a double-sector cell of 1.2-mm path length. [b] 12-mm path length. [c] Control with bovine serum albumin. Concentrations were all monitored by the absorbance at 414 nm. [d] Concentration was monitored by schlieren optics.

that the sedimentation coefficient of lumazine protein, which is 1.9 S in free solution, is increased slightly in the presence of *P. phosphoreum* luciferase.

In the presence of *V. harveyi* luciferase the M_r of lumazine protein is again increased. Subsequent to these measurements the decay of emission anisotropy experiments showed that this batch of luciferase was the one with only 14% binding competence (Figure 5). Therefore the sedimentation velocity experiments were made with the batch that showed the 43% binding competence. The sedimentation coefficient of luciferase alone was determined by schlieren optics as 4.6 S, an expected value corresponding to a molecular weight of 68 000 on an M_r vs. S plot [see Appendix 13 of Brewer et al. (1974)]. For lumazine protein in this solution of luciferase, the sedimentation coefficient determined by absorption optics monitoring the bound lumazine's absorption at 416 nm is 5.6 S, corresponding to a molecular weight of 92 000. This increase in sedimentation constant from 1.9 S is an additional direct demonstration of the attachment of one lumazine protein to the luciferase.

Discussion

The results obtained from the decay of anisotropy clearly indicate association of the lumazine protein isolated from *P. phosphoreum* with the luciferase from either *P. phosphoreum* or *V. harveyi*, since a longer rotational correlation time develops in the decay curve. These observations provide an unambiguous demonstration of the protein–protein interaction, because an intrinsic, natural chromophoric molecule, lumazine, serves as the reporter group. Thus, possible complications or perturbations arising by the use of externally introduced probes are avoided.

As is the case with the association of lumazine to its apoprotein, the two-protein system is well behaved, and a semiquantitative analysis of the equilibrium can be made, provided certain assumptions are accepted. From the relative weight (A_2) of the longer correlation time (ϕ_2) from the decay analysis and on the basis of the micromolar protein concentration of the luciferase, the tightest complex is obviously formed with *V. harveyi* luciferase, followed by *P. phosphoreum*, while *V. fischeri* luciferase does not bind lumazine protein at all. Corresponding effects also are found for the in vitro luciferase reactions: for *P. phosphoreum* and *V. harveyi*, lumazine protein shifts the bioluminescence spectrum, but it has no effect

in the *V. fischeri* reaction (Gast & Lee, 1978; Lee et al., 1981; Lee & Elrod, 1981). For *V. harveyi* luciferase the association can be studied under thermodynamically ideal conditions, i.e., low concentrations of interacting proteins in the millimolar to micromolar range, and this protein has therefore been employed in the more detailed study.

The method of analyzing the anisotropy data deserves some comment. Due to the inherent noise and the limited time span, often yielding less than one decade of decay, an unrestricted optimization gives widely scattered parameter values. On application of a constraint on the correlation times, the resulting fits show only a minor difference in quality with respect to the χ^2 criterion, but visually the fit appears to be equally good (Figure 4). From such an analysis physically meaningful results are obtained if, in addition, a simple binding equilibrium is assumed with two fluorescent species present: free and bound lumazine protein. The shorter correlation time has been obtained many times and can be reproducibly set at 23 ns. The longer correlation time can either be retrieved from the experimental decay when lumazine protein is completely bound or be calculated in a semiempirical way assuming the complex is a spherical particle with M_r 92 000. By this procedure we could unequivocally establish the concentrations of both fluorescent species from the relative amplitudes of the biexponential function.

In the completely bound complex the rotational correlation time is slightly longer than the 60 ns predicted but never approaches 100 ns, a value that takes into account the large amount of apparent hydration of lumazine protein itself. This discrepancy would be accounted for by a lesser degree of hydration of the complex. At pH 7 lumazine protein is a highly charged polyelectrolyte (pI = 5.0; Small et al., 1980) capable of attracting counterions as well as water molecules. It is conceivable that protein-bound water is released upon association and that other than hydrophobic interactions between luciferase and lumazine protein are important. This point will be discussed below.

An alternative explanation is that the correlation time of lumazine protein is somewhat longer than expected because it is nonspherical, whereas the complex more nearly approaches the shape of a spherical rotor. This proposal is hard to assess. With fluorescence depolarization a deviation from spherical symmetry will only be in evidence if the shape is a long prolate ellipsoid.

The correlation times of the complex may also be calculated from the ultracentrifuge data with the empirical formula $\phi = M_r\eta(\bar{v} + h)/(RT)$. By taking values for, respectively, η = 0.016 g cm^{-1}, M_r = 92 000, T = 275 K, $\bar{v} + h$ = 0.935 cm^3 g^{-1}, ϕ is calculated as 60 ns. On the other hand the Stokes' radius can be approximated by $R_h = (2.34 \times 10^{-3})M_r/S$, leading to R_h = 39 Å [cf. also Visser & Lee (1980)]. Substituting this value back in the Stokes–Einstein relation $\phi = 4\pi R_h^3\eta/(3RT)$, one arrives at ϕ = 17.6 ns, which is not in accordance with the experimental value. The fact that the Stokes–Einstein relation yields correlation times that are too short appears to be a general phenomenon, as a wide variety of biopolymers in solution give the same discrepancy between observed and calculated correlation times [for example, see Table 21-2 of Marshall (1978)]. The use of the empirical formula often leads to more congruent results.

The low value of the initial anisotropy, r_0, as observed both in lumazine protein and in the complex, might be a consequence of a very rapid depolarization (picosecond oscillation of the chromophore), which is nonresolvable with our apparatus. Lumazine in glycerol at 0 °C yields a considerably larger value of the steady-state anisotropy, $\langle r \rangle$ = 0.35, under comparable conditions of excitation and emission (Visser & Lee, 1980).

We can discuss only sketchily a number of other observations concerning the nature of this protein–protein interaction. A more complete answer to such questions will require a systematic study with larger amounts of these purified proteins than presently at our disposal. The first observation is the clear nonstoichiometry on the part of luciferase. This can be explained by assuming that a certain amount of the luciferase is incompetent for the binding of lumazine protein. Heterogeneity of binding has been encountered also in other protein–protein systems (Yphantis et al., 1978), and several explanations are proposed. There may be genetic variants present; e.g., one isozyme may possess a property rendering it incompetent for the interaction. Alternatively there may exist a conformer in solution that is not in rapid equilibrium with the competent structure. Luciferase displays a marked susceptibility to deactivation by proteases, and there is also a reactive cysteinyl residue that is essential for bioluminescence activity (Holzman & Baldwin, 1980a; Nicoli et al., 1974). Interpretation of kinetic analysis based on a simple model suggests that reaction of the cysteinyl or other residues, or the proteolysis, produces a proportion of the luciferase preparation that is inactive or has lost its stoichiometry in the bioluminescence reaction (Holzman et al., 1980; Welches & Baldwin, 1981).

There is not a clear relationship between the competence of luciferase for binding lumazine protein and its bioluminescence activity in the in vitro reaction. The two preparations of *V. harveyi* luciferase exhibit markedly different stoichiometries for binding lumazine protein. The effect of lumazine protein on the bioluminescence reaction with *P. phosphoreum* luciferase implies an interaction constant in the micromolar range (Lee et al., 1981), yet the binding between the two proteins is observed to be very weak (Table III). If only a few percent of this luciferase were competent for binding, the true K_d would be in the micromolar range, but this assumption is in discrepancy with the observed specific light yield of *P. phosphoreum* luciferase, which should be some measure of the content of active luciferase and which is actually greater than that from *V. harveyi*. The bioluminescence effect of the lumazine protein requires that all the active luciferase interacts with it. This apparent paradox is resolved by remembering that the bioluminescence is a kinetic process and the interaction constant is a kinetic parameter, not an equilibrium one. Preliminary observations suggest that lumazine protein functions by intercepting an energetic luciferase-bound intermediate in the scheme, a protein–protein, bimolecular reaction, and that the equilibrium binding to unreacted *P. phosphoreum* luciferase is in fact only weak, as is observed.

The effect of temperature (Figure 7) on the protein–protein equilibrium is similar to the effect on the ligand–protein dissociation for lumazine protein, where $\Delta H°$ = 28 kcal mol^{-1} (Visser & Lee, 1980). It appears that the protein–protein dissociation proceeds with very little enthalpy change and is "pulled" by the dissociation of the ligand.

Long-chain fatty aldehydes have no effect on the association of lumazine protein with luciferase. Aldehydes are bound to hydrophobic regions of the luciferase and protect the luciferase from inactivation by various agents (Nicoli & Hastings, 1974; Nicoli et al., 1974). Probably this hydrophobic region of the luciferase is not involved in the interfacial contact of lumazine protein and luciferase. Viswanatha et al. (1979) have studied

the interaction of dodecanal with the *V. fischeri* luciferase with ^{13}C NMR relaxation techniques. While they concluded that there was a strong interaction, their estimated rotational correlation time is much too short, 18 ns, and probably should be explained by independent motion of the aliphatic chain.

Finally, the observation that suggests itself as being fruitful to further experimentation is the phosphate effect. Phosphate strongly promotes the association between the two proteins. Calculations can be made from the data to show that the effect is not due to an enhancement of the actively binding content of the luciferase but is a genuine enhancement of the association constant. There is a clear parallel to the effect on other properties of the luciferase; for example, phosphate exerts a strong protective effect against the susceptibility of the luciferase activity to proteolysis by trypsin and chymotrypsin (Holzman & Baldwin, 1980b). For *V. harveyi* luciferase phosphate has an effect on its tertiary structure, reflected by changes in the far-UV circular dichroism (Holzman & Baldwin, 1980b).

One can only speculate about the real nature of the phosphate effect. The calculations presented suggest that there are considerable changes in hydration of the lumazine protein as it becomes complexed. Phosphate and other polyanions certainly have an important influence on protein hydration. According to Chothia & Janin (1975) electrostatic and van der Waals interactions are responsible for the complementarity of two protein surfaces, that is, their mutual recognition. Although rather unspecific, the driving force for the association is the hydrophobic interaction. The free energy for the association is derived from the decrease in surface area accessible to water, which suggests that for a stable complex to be formed at least 1600 Å^2 should be buried (Chothia & Janin, 1975). These conditions are probably fulfilled in the *V. harveyi* luciferase–lumazine protein system. It will be of value to find if phosphate and other anions have an effect on the interaction of lumazine protein with the other luciferases since they have been also demonstrated to be protected by phosphate against proteolysis (Baldwin et al., 1979).

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