# Lumazine Protein from the Bioluminescent Bacterium *Photobacterium phosphoreum*. A Fluorescence Study of the Protein–Ligand Equilibrium†

Antonie J. W. G. Visser and John Lee*

ABSTRACT: The changes of fluorescence spectral distribution, polarization, and lifetime of the lumazine protein from *Photobacterium phosphoreum* can be interpreted in terms of an equilibrium between the protein and its dissociated prosthetic group 6,7-dimethyl-8-(1′-D-ribityl)lumazine. The equilibrium is rapidly attained, 1:1, and $K_d$ is $5 \times 10^{-8}$ M (4 °C, pH 7, 67 mM phosphate). A change in solution conditions like an increase in temperature or dilution or a decrease in pH or ionic strength favors dissociation of the ligand from the protein. The dissociation was confirmed by separating the free ligand by ultrafiltration, and the apoprotein was reconstituted with the authentic lumazine derivative. A van't Hoff analysis of the dissociation constant allows evaluation of the thermodynamic parameters: $\Delta H°$ = 28 kcal/mol and $\Delta S°$ = 67 eu. By analogy to published results on the binding of FMN to apoflavodoxin, the contribution of hydrophobic interaction and of hydrogen bonding to the binding enthalpy can be estimated. The decay of the emission anisotropy of lumazine protein following a 0.5-ns laser pulse excitation can be fitted with a single correlation time characteristic of a 30000-dalton spherical protein.

The bioluminescent bacterium *Photobacterium phosphoreum* is an overproducer of a novel protein containing a highly fluorescent prosthetic group, 6,7-dimethyl-8-ribityllumazine (Koka & Lee, 1979). This lumazine protein is apparently functional in the bioluminescence process since it has the same fluorescence spectral distribution as the bioluminescence and it has kinetic and spectral effects on the in vitro reaction with luciferase (Gast & Lee, 1978).

The fluorescence spectrum of a solution of lumazine protein depends somewhat on its concentration. For instance, Gast & Lee (1978) measured the spectral maximum at 474 nm at 17 μM, shifting to around 490 nm below 1 μM, and they suggested that a dissociation of the fluorophore from the protein was responsible for this change. The free lumazine derivative (Lum)[1] is efficiently fluorescent with a maximum at 491 nm (Koka & Lee, 1979). It is also of interest that this same red shift is found between the spectral maxima of the in vivo bioluminescence and that from the in vitro reaction when $FMNH_2$, $O_2$, and dodecanal are the substrates.

Advantage has been taken of this apparent dissociation to effect a separation of the protein from its prosthetic group under mild conditions to allow the complete chemical structural characterization of the lumazine derivative (Koka & Lee, 1979).

In this present work we take advantage of fluorescence techniques to describe the interaction between the protein and the lumazine. Since both free and bound ligands are highly fluorescent, the system lends itself well to the application of fluorescence polarization and lifetime techniques. From the decay of the emission anisotropy of lumazine protein, the Stokes radius is calculated and found to be nearly consistent with the molecular weight of 22000 measured by other methods (Gast & Lee, 1978).

The binding is consistent with a simple 1:1 equilibrium and is rapidly attained. The spectral properties of the system are found to be so well-behaved that lumazine protein may provide a good experimental model for a general study of ligand–protein interactions.

## Materials and Methods

The procedure for the isolation and purification of the (blue fluorescence) lumazine protein from *P. phosphoreum* has been described elsewhere (Lee & Koka, 1978). 6,7-Dimethyl-8-(1′-D-ribityl)lumazine was a gift of Dr. W. Pfleiderer, University of Konstanz. The concentrations were calculated from the extinction coefficients, in which it was assumed that for lumazine protein $\epsilon_{414}$ is the same as that for the lumazine $\epsilon_{406}$, 10300 $M^{-1}$ $cm^{-1}$ (P. Koka and J. Lee, unpublished results; Maley & Plaut, 1959). All chemicals were of analytical grade. Absorption and fluorescence spectra were measured as described (Gast & Lee, 1978; Wampler, 1978). Polarization of the fluorescence ($p$) was measured with the apparatus described earlier (Visser et al., 1974), but with photon-counting electronics in the detection system, which permitted us to measure $p$ accurately even at very low concentrations of reactants (>10 nM). For these dilute samples corrections were applied for the background measured under the same conditions on a buffer solution during the same time interval. The determination of $p$ was essentially similar to that described by Jameson et al. (1978). Usually excitation was performed at 410 nm and emission was viewed through Schott KV 470 cutoff filters. Fluorescence lifetimes were determined with a phase fluorometer operating at 15 MHz ($\lambda_{exc}$ = 410 nm, emission through Schott KV 470) (Visser & Müller, 1980) or were evaluated from experimental fluorescence decay after excitation with picosecond pulses from a mode-locked Ar ion laser ($\lambda_{exc}$ = 458 nm, emission through Schott KV 470 and Balzers 501-nm interference filter) (Visser & van Hoek, 1979). The high repetition rate of the mode-locked Ar ion laser (76 MHz) was reduced to 297 kHz with an electrooptic modulator (Model 28, Coherent Associates, Inc.) (A. van Hoek and A.

† From the Department of Biochemistry, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands (A.J.W.G.V.), and the Bioluminescence Laboratory, Department of Biochemistry, University of Georgia, Athens, Georgia 30602 (J.L.). *Received March 18, 1980.* Work was supported by National Science Foundation PCM 79-11064, National Institutes of Health GM-19163, NATO Grant 1912, and the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

[1] Abbreviations used: Lum, 6,7-dimethyl-8-ribityllumazine; $P_i$, phosphate; ANS, 1-anilino-8-naphthalenesulfonate.

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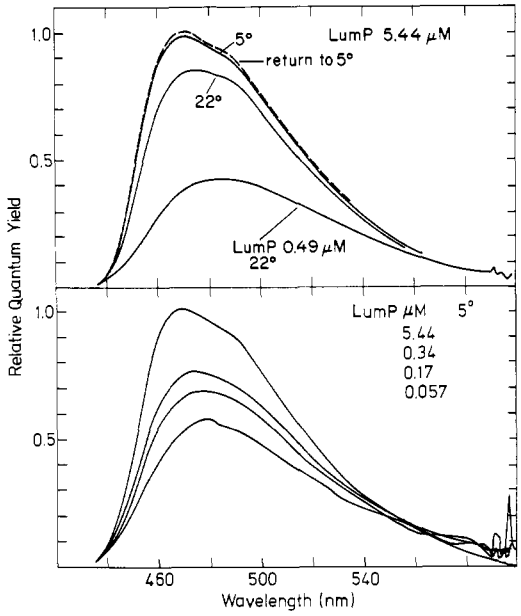


FIGURE 1: Fluorescence spectra of Lum*P* ($\lambda_{exc}$ = 410 nm). (Top) Effect of temperature in 67 mM $P_i$, pH 7.0, and 5.44 $\mu$M Lum*P*. When diluted 11× in water, fluorescence red shifts further. (Lower) Effect of concentration; all in 67 mM $P_i$, pH 7.0, and 5 mM mercaptoethanol, 5 °C.

J. W. G. Visser, unpublished results). The fluorescence decay data were analyzed by using a nonlinear least-squares method as described by Grinvald & Steinberg (1974). Measurement of the decay of the emission anisotropy and the inherent data analysis were performed as described by Visser & van Hoek (1979). The time-dependent anisotropy for a spherically shaped protein has the form

$$A(t) = A_0 \exp(-t/\phi) \quad (1)$$

where $\phi$ is the rotational correlation time and $A_0$ is the limiting anisotropy at $t = 0$. For a spherical protein the correlation time is related to the molecular Stokes radius $R_h$ by

$$\phi = \frac{4\pi R_h^3 N\eta}{3kT} = N\frac{V\eta}{kT} \quad (2)$$

where $\eta$ is the viscosity, $k$ is Boltzmann's constant, $N$ is Avogadro's number, $T$ is the absolute temperature, and $V$ is the molecular volume. The Stokes radius $R_h$ can be estimated from the molecular weight ($M_r$) and sedimentation coefficient ($s$) by assuming a specific volume ($\bar{v}$) of 0.735 cm$^3$/g for the protein (Pochon et al., 1978):

$$R_h\ (\text{Å}) = (2.34 \times 10^{-3})\frac{M_r}{s} \quad (3)$$

On the other hand, the correlation time for a spherical protein can be expressed as a function of molecular weight, specific volume, and degree of hydration ($h$, usually taken as 0.2 cm$^3$/g):

$$\phi = M_r\frac{(\bar{v} + h)N\eta}{kT} \quad (4)$$

## Results

It was noted by Gast & Lee (1978) that the fluorescence maximum of the (blue fluorescence) lumazine protein could be affected by dilution and also by a variety of mildly denaturing conditions, such as temperature, pH, and ionic strength. Figure 1 shows that dilution, increase in temperature, or decrease in the phosphate ion ($P_i$) concentration in the buffer all act in the same way to red shift the fluorescence maximum of the lumazine protein solution with a decrease in fluorescence quantum yield. At 0.5 $\mu$M concentration of lumazine protein, 22 °C, 7 mM $P_i$ (Figure 1, top), the fluorescence distribution is the same as that of free 6,7-dimethyl-8-ribityllumazine (Koka & Lee, 1979). A simple explanation is that increase in temperature or decrease in concentration or ionic strength all cause dissociation of the prosthetic group from the protein. Reversibility is indicated by the fact that when the temperature is changed from 5 to 22 °C and then back to 5 °C, identical fluorescence spectra are obtained (Figure 1, top).

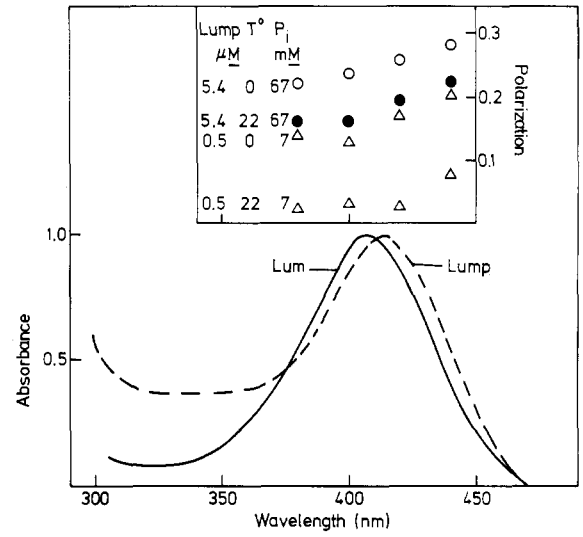


FIGURE 2: Absorption spectra of Lum (—) and a Lum*P* solution containing in addition a product absorbing at 320 nm (---) normalized to the same scale. The inset shows the degree of polarization ($p$) plotted as a function of excitation wavelength at the different solution conditions indicated.

At higher concentrations of lumazine protein the fluorescence distribution is unchanged from that at 5.44 $\mu$M, so this evidently is the fluorescence of the holoprotein. Intermediate spectra in Figure 1 can be satisfactorily matched by summation of the fluorescence distributions of free and bound ligand, but the observed intensity of fluorescence of the dissociated species is lower than expected, due to the lability, possibly photochemical, of the Lum under dilute conditions.

A more reliable method of measuring the dissociation constant should be by changes in the polarization of fluorescence since this is contributed to only by protein-bound Lum. Figure 2 shows that the solution conditions allowing the fluorescence red shift also cause the polarization to decrease, from above 0.2 at 5.4 $\mu$M to 0.02 at 0.5 $\mu$M. Gast & Lee (1978) measured a polarization of 0.17 for their preparation. All their fluorescence measurements were made at 23 °C, 50 mM $P_i$, with excitation at 415 nm and a concentration of lumazine protein of 6 $\mu$M, now calculated from the correct extinction coefficient of the chromophore since its structure is now known. Under similar solution conditions this value of 0.17 is seen to be in good agreement with the data of Figure 2. This polarization, however, is not a maximum so evidently the lumazine protein was partially dissociated under the conditions used by Gast & Lee (1978).

Figure 2 also compares the absorption spectrum of free 6,7-dimethyl-8-ribityllumazine with that of the lumazine protein preparation used in this present study. P. Koka and J. Lee (unpublished results) have noted that lumazine protein prepared using the improved method of purification including Blue Sepharose affinity chromatography (Lee & Koka, 1978;

Small, Koka, and Lee, unpublished results) shows no shoulders on the visible absorption band. These were evident in the preparation of Gast & Lee (1978) and probably came from a flavoprotein impurity. The preparations of P. Koka and J. Lee (unpublished results), however, show low absorbance in the 300–350-nm region, comparable to that seen for the free Lum in Figure 2. Figure 2 shows a higher contribution in this range for some lumazine protein solutions used in the present studies, and this is found to increase as the solutions age, particularly in the absence of added 2-mercaptoethanol (Lee & Koka, 1978). It is due to the formation of a product from the lumazine which absorbs at 320 nm and fluoresces with a maximum around 420 nm (Lee & Koka, 1978). It does not reach a level which can interfere with the fluorescence results reported here.

The polarization ($p$) of Lum in aqueous solution is as expected negligibly small. In glycerol at 0 °C, the $p_0$ is 0.450 ± 0.005, independent of excitation wavelength (350–450 nm) and concentration (1–20 μM). Figure 2 shows, however, that for bound Lum, $p$ increases as the wavelength of excitation is changed from 360 to 440 nm, and this increase is proportionally more at the lowest concentration and condition where the lumazine protein is largely dissociated. Such a wavelength-dependent polarization is often taken as evidence for more than one electronic transition contributing to an overall absorption envelope, but this is obviously explained more simply here by the dissociation. At the longer wavelengths the absorption spectra in Figure 2 show that the bound Lum contributes proportionally more to the total absorbance than the free Lum and this will become more important to the polarization measurement as the lumazine dissociates.

For excitation at the isosbestic point (410 nm) it is evident that the average polarization can be used to reliably measure the degree of dissociation (Chien & Weber, 1973). This technique is therefore adapted to measure the equilibrium parameters.

Gast & Lee (1978) measured the fluorescence quantum yield ($\phi_F$) for the lumazine protein to be 0.45 (23 °C, 50 mM $P_i$, 6 μM concentration, excitation 415 nm). Accompanying the fluorescence red shift on dilution, they found a reduction in $\phi_F$ and they also measured a corresponding decrease in fluorescence lifetime from 11.1 to 8.4 ns, respectively, using the single photon counting technique and an air gap spark for excitation at 358 nm.

These results are in good agreement with the results found here for the fluorescence lifetime by the phase and modulation technique, excitation 420 nm and modulation frequency 15 MHz, and by single photon counting, excitation 458 nm. Under the conditions where the lumazine is probably completely bound, 5.4 μM lumazine protein, 4 °C, 67 mM $P_i$, the phase method gives 12.3 ns and this drops to 11.1 ns at 1.4 μM lumazine protein, 22 °C, 67 mM $P_i$. By the single photon counting technique the lumazine protein has fluorescence lifetimes of 14.4 ns at 3 °C (Figure 3B) and 13.7 ns at 19 °C (not shown).

For the free Lum in aqueous solution, $\phi_F$ is 0.45 (5 μM, pH 7, 22 °C, 67 mM $P_i$). This was measured by reference to fluorescein (0.1 N NaOH, $\phi_F$ = 0.85) and quinine (1 N $H_2SO_4$, $\phi_F$ = 0.55) (Parker, 1968) and is independent of excitation wavelengths (360–410 nm) and temperature (5–25 °C). The fluorescence lifetimes by the single photon counting technique are 10.0 ns at 4 °C (Figure 3A) and 8.9 ns at 20 °C and that by the phase method (15 MHz) is 9.95 ns.

Lumazine protein has a $\phi_F$ = 0.59 (10 μM, pH 7, 5 °C, 50 mM $P_i$) measured against the same standards. It is independent of excitation wavelength (360–430 nm) but drops to 0.53 at 20 °C, slightly higher than the result for similar conditions published by Gast & Lee (1978). As already noted by P. Koka and J. Lee (unpublished results), the fluorescence quantum yield ratio between free and bound lumazine is the same as the ratio of the respective fluorescence lifetimes.

Table I: Effect of Dilution on the Degree of Polarization of the Fluorescence of Lumazine Protein (Lum$P$), 67 mM $P_i$, pH 7

| $[\text{Lum}P]_{\text{total}}$ (μM) | polarization at 4 °C | 10 °C | 16 °C | 21 °C |
|---|---|---|---|---|
| 5.44 | 0.213 | 0.214 | 0.200 | 0.182 |
| 2.72 | 0.214 | 0.196 | 0.185 | 0.133 |
| 1.36 | 0.199 | 0.162 | 0.152 | 0.106 |
| 0.69 | 0.183 | 0.142 | 0.130 | 0.069 |
| 0.35 | 0.152 | 0.113 | 0.066 | 0.025 |
| 0.175 | 0.145 | 0.126 | 0.047 | 0.020 |
| 0.09 | 0.058 | 0.047 | | |

If the equilibrium dissociation model is correct for the explanation of the fluorescence properties we have described above, then the decay of fluorescence intensity of a lumazine protein solution should exhibit two rate constants corresponding to the contributions of free and bound Lum fluorescence, as we have described above. The single photon technique is the preferred technique when dealing with such a complex situation, and Figure 3C shows unambiguously the development of a second faster decaying component of fluorescence as the solution conditions change to effect the red shift in the fluorescence seen in Figure 1. This provides direct evidence that the equilibrium model reaction is correct.

Parts A and B of Figure 3 show that the free and bound lumazines (4 °C) have a precisely exponential decay extending over several decades. Both raising the temperature and diluting the protein, parts C and D of Figure 3, allow the growing in of a shorter lifetime component nearly corresponding to free lumazine. The decay curves are more exactly fitted by a double-exponential function (cf. the deviations of parts C and D of Figure 3), and there is no clear evidence for a third contribution.

This would be an unambiguous technique for studying the equilibrium since both free and bound components are directly determined. However, the argon ion laser has appropriate emission wavelengths only at 458 and 364 nm, the extreme edges of the absorption band, and this leads to an amount of imprecision in determining the two contributions. Inspection of Figure 2 reveals that at 458 nm, excitation of bound lumazine is predominant. Therefore, the contribution of bound lumazine is overestimated somewhat in the decay of fluorescence measured at 505 nm, which corresponds with a more or less isoemissive region. Thus, the polarization of fluorescence excited at 410 nm is to be preferred as the more precise method. Nevertheless, the decay data of Figure 3 allow a rough estimate of the equilibrium dissociation constant and enthalpy as $K_d \approx 0.1$ μM (0 °C) and $\Delta H \approx 20$ kcal/mol, in satisfactory agreement with the results from polarization to be described next and also with the preliminary estimate of $K_d \approx 0.1$ μM made by Gast & Lee (1978).

The concentration-dependent shift in the fluorescence spectrum of the lumazine protein solution in Figure 1 is seen in Table I to be accompanied by a change in the fluorescence polarization. This change is also seen to be temperature dependent. All of the following results were made by a least-squares linear regression analysis. A reciprocal plot of polarization against concentration at each of the temperatures gives an estimate of $p_b$ as 0.23, the same at each temperature. This is the average polarization of bound lumazine protein

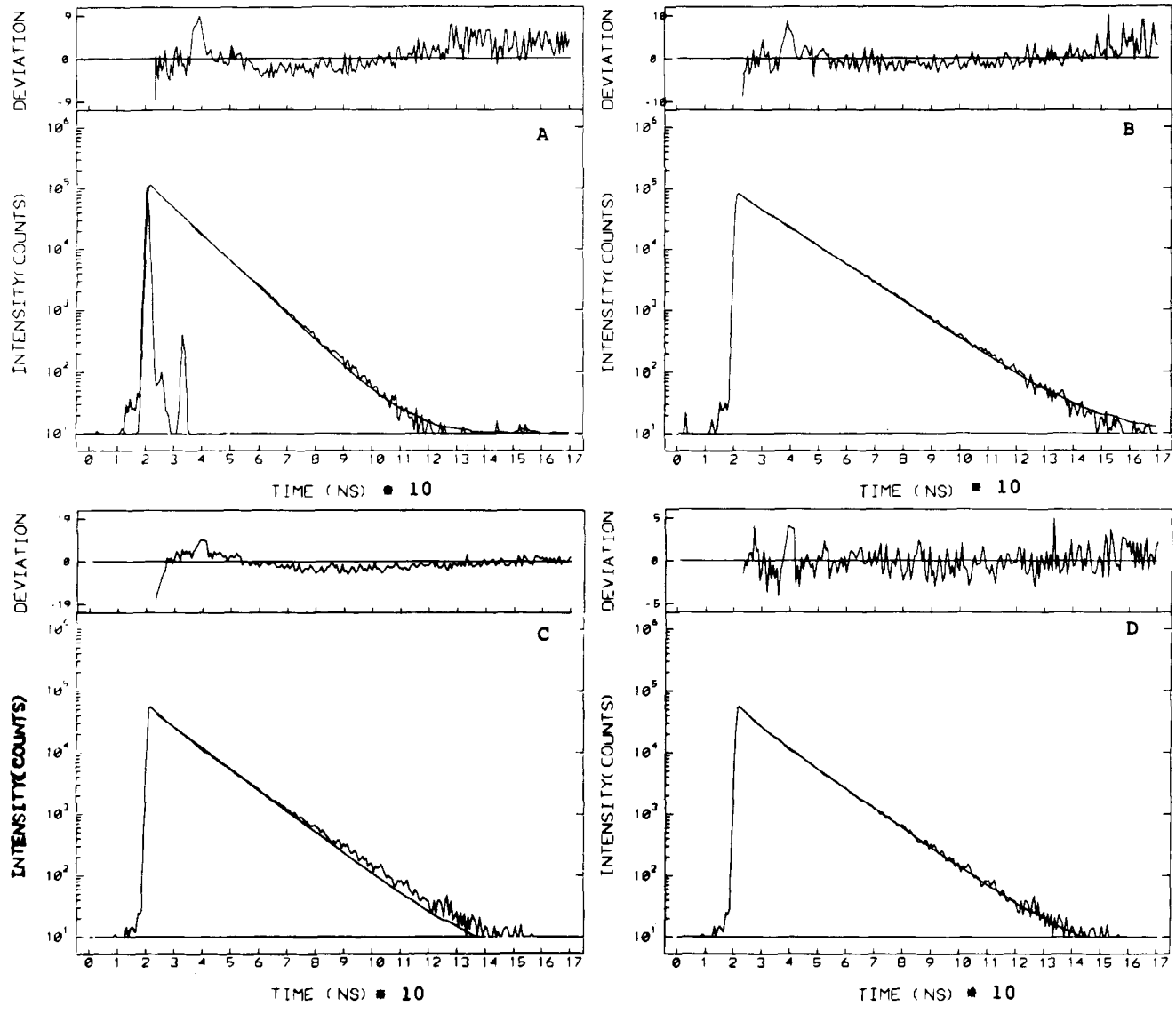


FIGURE 3: Fluorescence decay profiles of Lum and Lum*P*. (Top) Lum (A) and 5.0 $\mu$M Lum*P* (B) at 4 °C. (Lower) 1.0 $\mu$M Lum*P* at 20 °C (C and D). The following traces can be distinguished: laser pulse [only in (A), note the after pulse] and experimental [$F(t)$] and computed [$F_c(t)$] fluorescence decay assuming one (A, B, and C) or two (D) time constants. On top of each graph the residual values between calculated and experimental curves are presented. The deviation function in each channel $i$ is defined as $DV(i) = [F(i) - F_c(i)]/(F(i))^{1/2}$. The relevant parameters are as follows: (A) $\tau$ = 10.0 ns; (B) $\tau$ = 14.4 ns; (C) $\tau$ = 12.7 ns, $\chi^2$ = 13; (D) $\alpha_1$ = 0.68, $\tau_1$ = 13.9 ns, $\alpha_2$ = 0.32, $\tau_2$ = 6.0 ns, $\chi^2$ = 3. $\chi^2$ is given by $(1/N)\sum_{i=1}^{N}[DV(i)]^2$, where $N$ is the number of channels used. All compounds were in 67 mM $P_i$, pH 7.0, and 5 mM mercaptoethanol.

when excited at this wavelength and is used for all further calculations.

An analysis of these data and following tables by an equilibrium involving $n$ binding sites gives in every case a value of $n \leq 1$, so that all the results are therefore reanalyzed by making the assumption of a single binding site and the simple equilibrium

$$\text{Lum}P \rightleftarrows \text{Lum} + P \qquad (5)$$

where lumazine protein (Lum*P*) dissociates to free lumazine (Lum) and the apoprotein (*P*).

In Figure 1 the fluorescence red shift at higher temperature is seen to be fully reversible on lowering the temperature. So is the change in polarization as seen in Table III, lines 1–3, on changing the temperature from 4 °C to 22 °C to 4 °C. The dilution effect, line 6, is partially reversible, probably due to loss of substrates adsorbed in the membrane concentrator. The increase of polarization with increase in ionic strength, NaCl concentration, Table III, is also consistent with reversibility of the dissociation.

If the measured value of polarization is $p$, then it can be easily shown that

$$K_d = c[(I/I_f)(1 - p/p_b)]^2/[1 - (I/I_f)(1 - p/p_b)] \qquad (6)$$

where $c$ is the total concentration of free and bound Lum and $I/I_f$ is the ratio of the fluorescence intensities of Lum in the presence of protein to free Lum, respectively, under the excitation and fluorescence conditions of the experiment. This ratio varies experimentally between 1.6 and 1.1 depending on the conditions employed.

The results are given in Figure 4 in the form of a van't Hoff plot. On the basis of the simple equilibrium model eq 5, the $\Delta H°$ is estimated to be 28.0 ± 0.5 kcal/mol and the $\Delta S°$ as 67 ± 2 eu.

It has recently been suggested that authentic 6,7-dimethyl-8-ribityllumazine can reassociate with the apolumazine protein to produce a holoprotein with the same fluorescence and bioluminescence properties (Lee & Koka, 1979). An excess of the ligand was therefore added to a dilute solution

Table II: Influence of Ligand Addition on the Binding Equilibrium of Lumazine Protein (67 mM $P_i$, pH 7)

| $[Lum]_{total}$ ($\mu$M) | $[Lum]_{total}/[LumP]_{total}$ | $p^{4°C}$ | $\Phi^a$ | $p^{22°C}$ | $\Phi^a$ |
|---|---|---|---|---|---|
| 0.47 | 1.0 | 0.192 | 0.873 | 0.091 | 0.414 |
| 0.67 | 1.43 | 0.131 | 0.852 | 0.065 | 0.421 |
| 0.87 | 1.85 | 0.114 | 0.959 | 0.060 | 0.505 |
| 1.07 | 2.28 | 0.092 | 0.952 | 0.052 | 0.538 |
| 1.27 | 2.70 | 0.076 | 0.940 | | |

[a] $\Phi$ = degree of saturation ([bound protein]/[protein]$_{total}$).

Table III: Effect of Some Solution Changes on the Binding Equilibrium of Lumazine Protein

| [Lum*P*] ($\mu$M) | *p* | $K_d$ ($\mu$M) | *T* (°C) | $\Delta H$ (kcal/mol) | $P_i$ (mM) | pH |
|---|---|---|---|---|---|---|
| 4.7 | 0.217 | | 4 | | 67 | 7 |
| 4.7 | 0.155 | 0.58 | 22 | 22 | 67 | 7 |
| 4.7 | 0.203 | | 4 | | 67 | 7 |
| 0.47 | 0.091 | 0.39 | 22 | 33 | 67 | 7 |
| 0.47 | 0.192 | 0.01 | 4 | | 67 | 7 |
| <4.7 | 0.170 | | 4 | | 67 | 7 |
| 0.47 | 0.034 | 2.22 | 22 | | 67 | 6 |
| 0.47 | 0.113 | 0.22 | 4 | 21 | 67 | 6 |
| 0.47 | 0.112 | 0.22 | 22 | 34 | 67 | 8 |
| 0.47 | 0.201 | 0.005 | 4 | | 67 | 8 |
| 0.47 | 0.035 | 2.09 | 22 | 29 | 7 | 7 |
| 0.47 | 0.146 | 0.08 | 4 | | 7 | 7 |
| 0.82 | 0.043 | | 22 | | 67 | 7 |
| 0.82 | 0.044 | | 22 | | 67 + 0.1 M NaCl | 7 |
| 0.82 | 0.065 | | 22 | | 67 + 0.5 M NaCl | 7 |
| 0.82 | 0.089 | | 22 | | 67 + 1.0 M NaCl | 7 |
| 0.82 | 0.154 | | 4 | | 67 | 7 |
| 0.82 | 0.164 | | 4 | | 67 + 0.1 M NaCl | 7 |
| 0.82 | 0.170 | | 4 | | 67 + 0.5 M NaCl | 7 |
| 0.82 | 0.175 | | 4 | | 67 + 1.0 M NaCl | 7 |

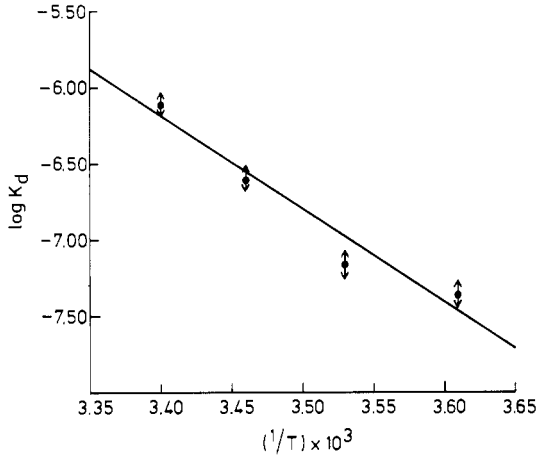


FIGURE 4: van't Hoff plot of dissociation constant ($K_d$) for dissociation of Lum from Lum*P*.

of lumazine protein, and the changes of polarization (Table II) were found to be consistent with the equilibrium (eq 5) being driven to the left-hand side by the excess ligand.

The results of Table II are again analyzed in the simple model (eq 5) and give $K_d$(4 °C) $\approx$ 0.1 $\mu$M, $K_d$(22 °C) $\approx$ 0.6 $\mu$M, and $\Delta H° \approx$ 25 kcal/mol. While consistent with the first set of results (Figure 4), there is not good agreement in the $K_d$ values (2–3×) and the reasons for this are not clear. Other batches of lumazine protein give the same polarization results as reported above. However, there are changes in the parameters as a solution ages, even at 5 °C in the presence of 2-mercaptoethanol, and also if it is put through a number of freeze–thaw cycles. These observations point to an instability of the apoprotein. The true values of $K_d$ then can be taken to be the lower values reported above, and confidence can be given to the thermodynamic parameters derived from them.

Table III lists the effect of some solution changes on the polarization values, interpreted again without detailed justification but for the purpose of discussion, in terms of $K_d$ and $\Delta H$. From pH 7 to 8 the values do not change significantly, but pH 6 causes the $K_d$ to increase markedly, equivalent for instance to a 4–22 °C temperature increase at pH 7. The same is seen at lowered phosphate ion concentration, while the binding becomes tighter when an increasing amount of NaCl is added. Thus, the lower pH or lower ionic strength favors dissociation and since the lumazine itself has no p$K$ in this region, this could result from a conformation of the apoprotein $P'$ favored by these conditions and unable to bind the ligand tightly:

$$LumP \rightleftarrows Lum + P \rightleftarrows P' \quad (7)$$

A direct test of the dissociation (eq 5) is membrane ultrafiltration of a lumazine protein solution which polarization data indicate is in a largely dissociated form. The prosthetic group should pass through a YM10 membrane (Amicon Diaflo) which retains molecules above 10000 daltons. A 2.05 $\mu$M lumazine protein solution in 5 mM Bis-Tris buffer, pH 7, and 1.6 mM 2-mercaptoethanol contained in an Amicon Stirred Cell with a YM10 membrane was concentrated from 50 to 20 mL by applying nitrogen pressure at 23 °C. Light was excluded because Lum is photosensitive. The absorption spectrum of the eluate is in Figure 5, upper full curve, and is seen to be the same as that for the authentic compound, dashed line, as judged by the wavelength position of the visible maximum, 406 nm, and the optical density ratio, 276:406. Below 275 nm interference by the 2-mercaptoethanol makes comparison inaccurate. The fluorescence maximum of the

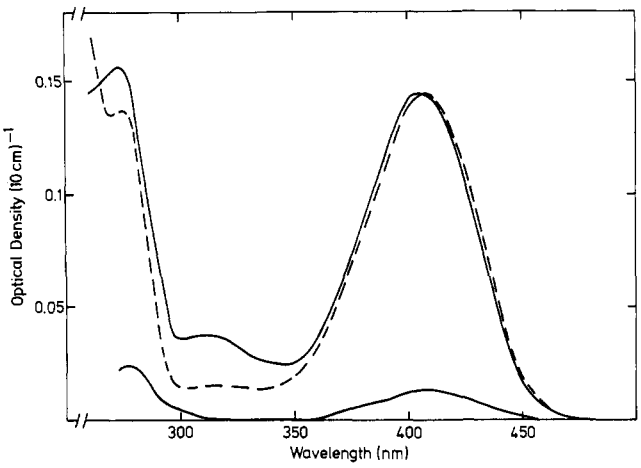


FIGURE 5: Absorption spectra (10-cm path length) of the eluate (30 mL) from ultrafiltration of lumazine protein at low ionic strength at 23 °C. Initial concentrations (50 mL) were 2.05 μM for the eluate absorbance in the top full curve and 0.186 μM for the lower full curve. Authentic 6,7-dimethyl-8-ribityllumazine has the same spectrum (dashed curve, 1.42 μM).

eluate excited at 420 nm is at 490 nm, the same as that of the authentic compound.

By use of a fluorescence assay at 290 → 340 nm to measure protein, the amount passing the YM10 membrane was 7% of the total. When lumazine protein is concentrated under higher ionic strength conditions at 5 °C, where the polarization measurements indicate that it is completely associated, less than 5% of the total lumazine or protein as assayed by fluorescence is found in the eluate.

The lower full curve in Figure 5 is the eluate absorbance after ultrafiltration under the same conditions of a lumazine protein solution of initial concentration 0.186 μM. About 95% of the Lum goes through the membrane in this case compared with 75% in the first experiment.

In Figure 5 the upper curve corresponds to a Lum concentration of 1.42 μM, from which $K_d$ is calculated to be 3.3 μM, a value in good agreement with that obtained from polarization, under similar conditions (Table III, line 11).

The solution which had started at 2.05 μM was further concentrated (23 °C) to 4 mL, and to this apoprotein was added 50 mL of a 2.0 μM solution of authentic 6,7-dimethyl-8-ribityllumazine in 0.1 M $P_i$, pH 7, and 1.6 mM 2-mercaptoethanol. After 2 days of incubation at 5 °C to encourage maximum recovery of the holoprotein, the solution was concentrated (5 °C) to 5 mL (YM10). By fluorescence assay about 30–40% of the lumazine and protein passed through the membrane. The concentrate had a fluorescence maximum at 470 nm characteristic of the bound lumazine (Gast & Lee, 1978).

These experiments therefore confirm both the reconstitution of the lumazine protein previously reported by Lee & Koka (1979) and the equilibrium model of eq 5.

The fluorescence lifetime and molecular weight of lumazine protein are just in the right range as to allow the decay of fluorescence emission anisotropy to be measured very precisely. Figure 6 shows the emission anisotropy decay at two temperatures to be a single exponential over the first decade. At room temperature Table I indicates that there is a certain amount of dissociation of this lumazine protein solution, yet the Stokes radius, 2.0 nm, calculated from the decay of emission anisotropy of bound lumazine (eq 2), is almost the same as that calculated from eq 3 at that temperature. It is important to notice that the limiting anisotropy $A_0$, i.e., the value of $A$ at $t = 0$, is distinctly lower at the higher temperature

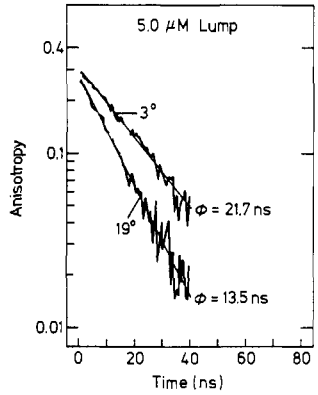


FIGURE 6: Decay of emission anisotropy of 5.0 μM Lum*P* at two temperatures. The noisy curves reflect experimental data points; the straight line is a nonlinear regression fit assuming a single correlation time. Values are indicated in the figure.

Table IV: Anisotropy Characteristics of Lumazine Protein

| $T$ (°C) | $A_0$ | $\phi$ (exptl) (ns) | $\phi^a$ (calcd) (ns) | $R_h{}^b$ (exptl) (nm) | $R_h{}^c$ (estd) (nm) |
|---|---|---|---|---|---|
| 19 | 0.25 | 13.5 | 8.7 | 2.0 | 2.5 |
| 3 | 0.30 | 21.7 | 14.5 | 1.9 | |

[a] Calculated from eq 4. [b] Obtained from eq 2. [c] Estimated from eq 3.

(3 °C, $A_0 = 0.30$; 20 °C, $A_0 = 0.25$). This confirms that a certain amount of Lum is dissociated, giving rise to a distinct initial depolarization. The correlation times indicate a somewhat higher molecular weight than expected, which might point to a higher degree of hydration. A survey of all results (experimental and calculated) is given in Table IV.

## Discussion

Fluorescence is a sensitive and precise technique for studying protein structure and interactions. For lumazine protein the fluorescent probe itself has the advantage of being the natural prosthetic group. It has the added features that it is efficiently fluorescent both on and off the protein and that the fluorescence lifetimes in the two states are of sufficient magnitude and difference so that they, and the resulting fluorescence polarization, can be measured with high precision.

It is also remarkable that in its fluorescence properties, lumazine and lumazine protein are very well-behaved. The decay of fluorescence is accurately fitted to a single rate extending over several decades (parts A and B of Figure 3). In almost every case reported hitherto, when a fluorophore binds to a protein its fluorescence decay, which is usually quite simple in free solution, usually becomes more complex. Heterogeneity of the binding site environment is often proposed to explain this, certainly a plausible explanation in the case of an artificial probe like ANS, which might be expected to find a variety of nonpolar regions on the protein surface. But for a natural coenzyme, FAD for instance, which would be expected to bind to a very specific site, the complexity in fluoresence decay is more difficult to explain (cf. also proteins containing a single tryptophan residue; Grinvald & Steinberg, 1976).

A departure from simple exponential decay under certain solution conditions of the lumazine protein becomes then immediately understandable in terms of the dissociation of the free fluorophore. The second component in the lifetime which now appears approaches that of the free lumazine, and this supports the validity of the equilibrium model proposed to explain the fluorescence shift observed.

The use of steady-state fluorescence polarization for the study of protein–ligand interactions has been discussed by Chien & Weber (1973). After establishment of the equilibrium model, the interpretation of the data becomes quite unambiguous. Only two states exist, free and bound lumazine, and there is no evidence for an intermediate state $BFP_{490}$, i.e., a bound lumazine with a fluorescence maximum at 490 nm. Gast & Lee (1978) proposed this intermediate since on dilution, which affected the fluorescence red shift and which reduced the fluorescence lifetime to 8.4 ns, the steady-state fluorescence polarization was still significant, around 0.1.

The present results suggest that this solution could still have contained a significant amount of undissociated lumazine protein to account for the polarization observed. In the flash method used for their lifetime measurement the excitation was from an emission line of nitrogen at 358 nm. Figure 2 shows that this would favor the excitation of unbound lumazine or the impurity flavoprotein and thus produce a lower average lifetime. It is to be noted that for the lumazine protein itself all three methods give the same value of fluorescence lifetime, 11–14 ns, for the three wavelengths of excitation.

A consequence of the simple behavior of the average fluorescence decay is that the emission anisotropy also decays with a single exponential (Wahl, 1975; Dale et al., 1977). This can be taken to mean that the lumazine protein is a spherical molecule although there is apparently some controversy over whether such a conclusion can be made too strongly (Small & Isenberg, 1977). Even when the lumazine protein solution is in a partly dissociated state there is no evidence for any gross change in the dimensions of the conjugated protein.

In general the factors which influence the binding of a prosthetic group to its apoprotein are of considerable interest. The present results are sufficiently accurate to show that there is considerable enthalpy involved in the dissociation, about 28 kcal/mol. It has been calculated that the gain of translational and rotational entropy favoring the dissociation of a molecule the size of the lumazine from the protein gives a $T\Delta S$ of 18 kcal/mol (Janin & Chothia, 1978). The entropy in the lumazine protein dissociation is consistent with this estimate, favoring dissociation by 28 − 10 = 18 kcal/mol ($\Delta H° - \Delta G° = T\Delta S°$).

Two types of intermolecular interactions can account for the 28 kcal/mol endothermicity. Raman studies of the lumazine protein (Irwin et al., 1980) show only minor interactions of the electronic system of the lumazine ring with the protein in the ground state. Therefore, in direct analogy to the structure of flavodoxins, where the ribityl tail of the FMN is found to be buried in the protein, it can be proposed that in lumazine protein (FMN after all is a benzalumazine derivative) the ribityl group is also buried and the hydrophobic portion remains exposed to solvent. Unlike flavodoxin, the lumazine ribityl group has no phosphate (Koka & Lee, 1979) so that no ionic forces can contribute to the interaction. It is possible that the ribityl OH groups form tighter hydrogen bonds to acceptors in the binding site than to $H_2O$, and, since there are four OH available, 10 kcal/mol from this source would be a reasonable estimate.

The other interaction which is believed to provide a major driving force to the association is the reduction in surface area accessible to solvent (Janin & Chothia, 1978). Since the structure of lumazine protein is not known, we have to resort again to the analogy of flavodoxin. In their Table I, Janin & Chothia calculated 492 and 244 Å$^2$ for the surface area buried in the complex contributed by coenzyme (FMN) and protein, respectively. In the case of lumazine protein, we can guess that at least one-half of the FMN value, i.e., 250 Å$^2$, would be contributed by the lumazine and the same amount again by the protein, a total of 500 Å$^2$. About 25 cal/mol is available for each Å$^2$, a total of 12 kcal/mol. The addition of this to hydrogen bonding satisfactorily accounts for the 28 kcal/mol enthalpy actually observed.

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