# Spectral Properties and Function of Two Lumazine Proteins from *Photobacterium*†

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*Received October 21, 1983; Revised Manuscript Received August 22, 1984*

ABSTRACT: The spectral properties are compared for two 6,7-dimethyl-8-ribityllumazine proteins from marine bioluminescent bacteria, one from a psychrophile, *Photobacterium phosphoreum*, and the other from a thermophile, *Photobacterium leiognathi*. The visible spectral properties, which are the ones by which the protein performs its biological function of bioluminescence emission, are almost the same for the two proteins: at 2 °C and 50 mM $P_i$, pH 7, fluorescence quantum yield $\phi_F$ = 0.59 and 0.54, respectively; fluorescence lifetime $\tau$ = 14.4 and 14.8 ns, respectively; fluorescence maxima, both 475 nm; absorption maximum, 417 and 420 nm, respectively; circular dichroism minima at around 420 nm, both $-41 \times 10^3$ deg cm$^2$ dmol$^{-1}$. The ligand binding sites therefore must provide very similar environments, and arguments are presented that the bound ligand is relatively exposed to solvent. The dissociation equilibrium was studied by steady-state fluorescence polarization. The thermophilic protein binds the ligand with $K_d$ (20 °C) = 0.016 $\mu$M, 10 times more tightly than the other protein [$K_d$ (20 °C) = 0.16 $\mu$M]. The origin of the binding difference probably resides in differences in secondary structure. The tryptophan fluorescence spectra of the two proteins are different, but more significant is an observation of the decay of the tryptophan emission anisotropy. For the psychrophilic lumazine protein this anisotropy decays to zero in 1 ns, implying that its single tryptophan residue lies in a very "floppy" region of the protein. For the other protein, the anisotropy exhibits both a fast component and a slow one corresponding to rotation of the protein as a whole. This suggests that in the thermophilic protein the tryptophan region is held more rigidly. In both proteins, however, the ligand exhibits no independent mobility, as its rotational correlation time (respectively 19.5 and 17.5 ns, 2 °C) corresponds to the rotation of a sphere of hydrated $M_r$ ~30 000.

Lumazine protein (LumP)[1] is a blue fluorescence protein that has now been identified in several strains of *Photobacterium phosphoreum* and *Photobacterium leiognathi* (Koka & Lee, 1979; Lee, 1982; Vervoort et al., 1983). It is probably common within the genus *Photobacterium* (Vervoort et al., 1983; O'Kane et al., 1985). The ligand 6,7-dimethyl-8-ribityllumazine (Lum) is highly fluorescent both on and off the protein (Visser & Lee, 1980; Lee et al., 1981). The spectral properties of LumP have therefore been used to advantage to follow its purification, to allow for its tentative identification, and to study the protein–ligand equilibrium LumP $\rightleftharpoons$ Lum + P, where P is the apoprotein (Visser & Lee, 1980). By measuring the fluorescence polarization of *P. phosphoreum* LumP solutions, Visser & Lee (1980) evaluated $K_d$ as >0.05 $\mu$M, at 2 °C and 67 mM $P_i$. They showed that the dissociation was favored by lower ionic strength and higher temperature.

The LumP studied by Visser & Lee (1980) was one isolated from psychrophilic strain A13 of *P. phosphoreum*. This bacterium is grown routinely at 12 °C (Lee & Koka, 1978) and occurs in the oceans at an ambient temperature around 5 °C (FitzGerald, 1978; FitzGerald & Lee, 1978). A recent report identified a LumP from *P. leiognathi* strain S1, which is grown around 30 °C (Vervoort et al., 1983). This strain is relatively thermophilic for a marine bacterium. It was observed for the "*P.l.*" LumP that the ligand was much less easily dissociated from the protein in the course of isolation than had been the case for the "*P.p.*" type. On the other hand, the physical and spectral properties of the two LumPs were nearly the same (O'Kane & Lee, 1985a,b; O'Kane et al., 1985).

The development of new purification protocols has now made available homogeneous preparations of both LumPs in quantity (O'Kane et al., 1985). A systematic study of their spectroscopic properties is presented here. It is of course their fluorescence property that has the biological function. Some preliminary data on the *P.p.* LumP have been presented before (Koka & Lee, 1981).

## Materials and Methods

The origin of the bacteria *P. phosphoreum* strain A13 and *P. leiognathi* strain A2D (a substrain of S1) and the methods for obtaining the homogeneous LumPs have been described previously (O'Kane et al., 1985). For all measurements of LumP a "standard buffer" was used: 50 mM sodium phosphate and 5 mM 2-mercaptoethanol, pH 7.0. Lum and 6-methyl-7-oxo-8-ribityllumazine were gifts of Professor H. C. S. Wood (University of Strathclyde). All other chemicals used were of the best commercial grades.

† This work was supported by National Institutes of Health Grant GM28139, NATO Grant 835/83, the University of Georgia Research Foundation, 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.).

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[1] Abbreviations: Lum, 6,7-dimethyl-8-ribityllumazine; LumP(s), lumazine protein(s); *P.l.*, *Photobacterium leiognathi*; *P.p.*, *Photobacterium phosphoreum*; P, apoprotein; E, bacterial luciferase; $\phi_F$, fluorescence quantum yield; $\tau$, fluorescence lifetime; $\phi$, rotational correlation time; $A$, emission anisotropy amplitude; FMN, riboflavin 5′-phosphate; $P_i$, inorganic phosphate.

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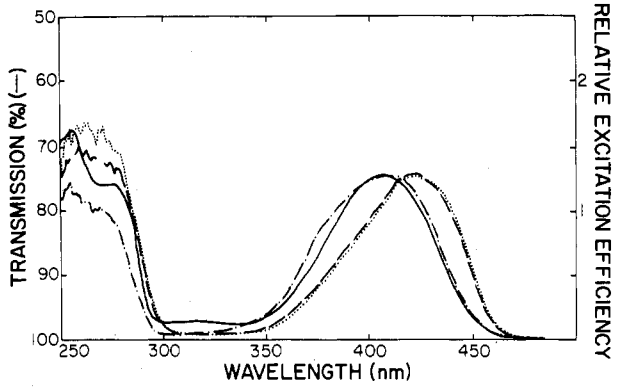


FIGURE 1: Corrected excitation spectra for fluorescence at 500 nm and at 2 °C: *P.p.* LumP, 5 μM (--); *P.l.* LumP, 5 μM (···); Lum, 10 μM (-·-). All spectra are normalized to the same height as the visible band of the transmission spectrum of Lum, 10 μM (—).

The concentrations of LumP were determined by absorbance (O'Kane et al., 1985): *P.p.*, $\epsilon_{417}$ = 10 300 $M^{-1}$ $cm^{-1}$; *P.l.*, $\epsilon_{420}$ = 10 100 $M^{-1}$ $cm^{-1}$. Absorbance and corrected fluorescence spectra were measured as described previously (Lee, 1982; Gast & Lee, 1978). For Figure 5 however, since correction factors below 350 nm had not been made previously in the calibration with reference to the National Bureau of Standards Standard Lamp (Wampler, 1978), the factors were estimated by logarithmic extrapolation of the NBS-derived correction factor curve from 350 nm down to 300 nm. For correction of the excitation spectra, calibration of the xenon lamp intensity impinging on the sample was made for the region 300–500 nm by comparing the technical excitation spectrum to the absorption spectrum of dilute solutions of FMN (pH 7) and quinine sulfate (1 N $H_2SO_4$) and for the region 250–400 nm by using a rhodamine B (propylene glycol, 2 °C) quantum counter (Parker, 1968). There was no significant polarization effect measurable on the excitation spectrum.

The steady-state fluorescence polarization and the decays of fluorescence emission intensity and anisotropy were measured as described elsewhere (Visser & Lee, 1980, 1982; Visser & Santema, 1981; van Hoek et al., 1983). Fluorescence decay curves were fitted to exponential functions according to nonlinear least-squares procedures (Grinvald & Steinberg, 1974; Visser & van Hoek, 1979). Anisotropy decay curves were analyzed with a parameter-fitting procedure to be described elsewhere (Visser et al., 1985). Circular dichroism was obtained on a Jasco J-20 CD spectrometer with the help of Dr. D. E. Edmondson, Emory University (Atlanta, GA). Cells of 1-cm path length were used at room temperature, and the spectra are corrected to solvent base line.

## RESULTS

Figure 1 is a comparison of the relative fluorescence excitation spectra (corrected) for the two LumPs and for the free ligand Lum. The transmission spectrum of Lum is also drawn because this, rather than the absorption spectrum, is the appropriate one to be compared directly to the excitation spectrum. All spectra are normalized to give the same height for the band at 410–420 nm. Technical difficulties limit the accuracy for obtaining correction factors for instrumental excitation spectra. Nevertheless, there is satisfactory agreement in Figure 1 between the excitation and transmission spectra in the visible region for Lum. Although the width of the excitation band is greater than that of the transmission band, the excitation maximum at 410 nm is only slightly different from the transmission minimum (i.e., absorption maximum) at 408 nm. However, it is significant that the

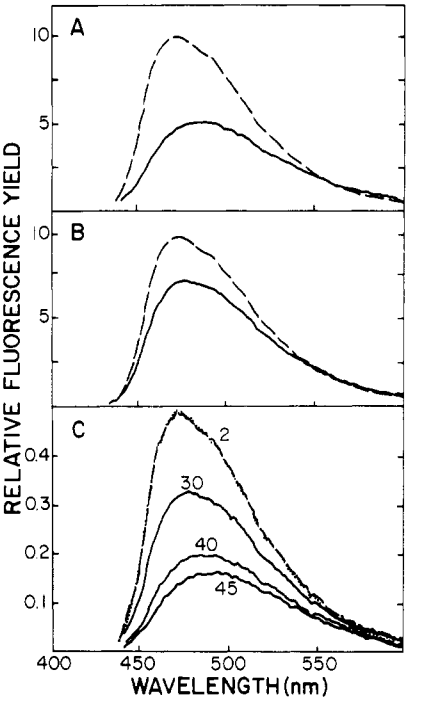


FIGURE 2: Changes in LumP concentration and temperature both affect its fluorescence spectrum due to the dissociation of the ligand. Excitation was 410 nm. (A) *P.p.* LumP: 7.7 μM, 2 °C (--); 0.33 μM, 20 °C (—). (B) *P.l.* LumP: 7.7 μM, 2 °C (--); 0.33 μM, 20 °C (—). (C) *P.l.* LumP: 0.33 μM, 2 °C (--); 30, 40, and 45 °C (—); returned to 2 °C and measured after 3 h (···).

280/410 ratio of heights is lower for the excitation, 0.7, than for the transmission spectrum, 0.92. Also, the minor bump around 320 nm in the transmission spectrum does not show itself in the excitation. This 320-nm absorbance probably arises from spontaneous oxidation of Lum to the 6-methyl-7-oxo derivative with a fluorescence maximum at 420 nm, as its intensity increases with time with a corresponding loss of Lum 420- → 490-nm fluorescence intensity.

The excitation spectra of two LumPs (Figure 1) are in good correspondence to their transmission spectra (not shown) in the visible region. However, the height ratio 280/420 is much lower than the 2.2 ratio observed in the transmission spectrum (O'Kane et al., 1985). (The transmission and absorption spectra have the same shape if the absorbance is below about 0.1, which it was for the solutions measured in Figure 1.) The excitation ratios are as follows: *P.p.* LumP, 1.05; *P.l.* LumP, 1.14. They are different between themselves, higher than for free Lum, and higher than the Lum transmission ratio.

The fluorescence spectra of the two LumPs can be compared in parts A and B of Figure 2 (dashed curves). Their fluorescence spectra are nearly identical with maxima both at 475 nm and a consistently appearing shoulder in the neighborhood of 490 nm, probably from a vibronic contribution. The dashed spectra in panels A and B are for a concentration of 7.7 μM and 2 °C. On 20× dilution and on bringing to room temperature (solid lines), the fluorescence red shifts and decreases in intensity. This effect has been explained before as due to dissociation of Lum, which has a fluorescence maximum at 490 nm and a decreased $\phi_F$ of 0.45 (20 °C) (Visser & Lee, 1980). The effect is much more marked for *P.p.* LumP than for *P.l.* LumP, almost complete dissociation being effected in the former case, while in the latter the ligand remains mostly bound.

The lowest panel in Figure 2 shows that for *P.l.* LumP at this same concentration, 0.33 μM, the temperature has to be about 45 °C before the Lum is completely dissociated and the

Table I: Effect of Solution Changes on Steady-State Fluorescence Polarization ($p$) of *P.l.* LumP (31 °C and 50 mM $P_i$, except As Noted)

| [total LumP] ($\mu$M) | $p$ | condition |
|---|---|---|
| 7.7 | 0.183 | |
| 0.78 | 0.145 | |
| 0.31 | 0.117 | |
| 0.175 | 0.092 | |
| 0.058 | 0.065 | |
| 0.31 | 0.095 | 10 mM $P_i$ |
| 0.31 | 0.173 | 500 mM $P_i$ |
| 0.31 | 0.152 | 500 mM KCl |
| 0.31 | 0.170 | 1000 mM KCl |
| 0.44 | 0.204 | 20 °C |
| 0.30 | 0.065 | 40 °C |

Table II: *P.l.* LumP Equilibrium Dissociation Constant and Steady-State Fluorescence Polarization ($p_b$)[a] (50 mM $P_i$, pH 7)

| $T$ (°C) | $p_b$ | $K_d$ ($\mu$M) | $T$ (°C) | $p_b$ | $K_d$ ($\mu$M) |
|---|---|---|---|---|---|
| 2 | 0.29 | | 31 | 0.17 | 0.073 |
| 10 | 0.27 | | 35 | 0.15 | 0.2 |
| 20 | 0.23 | 0.016 | 40 | 0.10 | 0.2 |
| 25 | 0.20 | 0.044 | 20 | 0.202[b] | 0.16[b] |

[a] Calculated from $p^{-1} = p_b^{-1} + A[\text{LumP}]^{-1}$; $A$ = a constant. [b] Remeasurement of *P.p.* LumP (previously $p_b = 0.204$, $K_d = 0.56$ $\mu$M; Visser & Lee, 1980).

dissociation is completely reversible, since returning this 45 °C solution to 2 °C (dotted line) produced a fluorescence identical with the starting one. About 20 min was allowed for equilibration at each temperature except for the last (dotted line, 3 h). Equilibrium appeared to be established in these times. For a buffer containing a lower phosphate concentration (5 mM) at 20 °C, the fluorescence of *P.l.* LumP (0.33 $\mu$M) becomes very similar to the one at 30 °C in panel C, indicating about 30% free Lum. At 200 mM $P_i$ (20 °C) and again 0.33 $\mu$M LumP the fluorescence maximum was blue shifted to 476 nm, indicating that the Lum was completely bound again. An equivalent 30% dissociation was also produced by pH 5 or 8 (50 mM $P_i$, 20 °C).

The *P.l.* LumP was found to have its maximum $\phi_F$ at pH 6.5 (2 °C). At pH 7 as in Figure 2B, the $\phi_F$ is about 5% lower. By comparison with *P.p.* LumP at pH 7 where $\phi_F = 0.59$ (Visser & Lee, 1980) it is found that, for *P.l.* LumP at pH 6.5 (2 °C), $\phi_F$ also is 0.59.

Measurement of the steady-state fluorescence polarization ($p$) of a LumP solution has been shown to be a precise and unambiguous technique for defining the equilibrium (Visser & Lee, 1980). Free Lum has $p = 0$, and when bound ($p_b$), it has a higher value depending on conditions. Table I shows polarization data from a representative experiment with *P.l.* LumP. Dilution decreases $p$ due to a decrease in bound Lum from the equilibrium dissociation. At 0.31 $\mu$M, an increase in ionic strength by added $P_i$ or KCl (it is not specific) increases $p$, i.e., favors association. The same qualitative behavior has been previously shown for *P.p.* LumP.

Extrapolation of $p$ to infinite concentration yields the value of $p_b$, and these are collected in Table II. The temperature dependence of $p_b$ is due to the change in viscosity of the solvent. Again from Visser & Lee (1980) (Chien & Weber, 1973), the equilibrium dissociation constant can be calculated from the relationship

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

where $c$ is the total concentration of free and bound Lum and

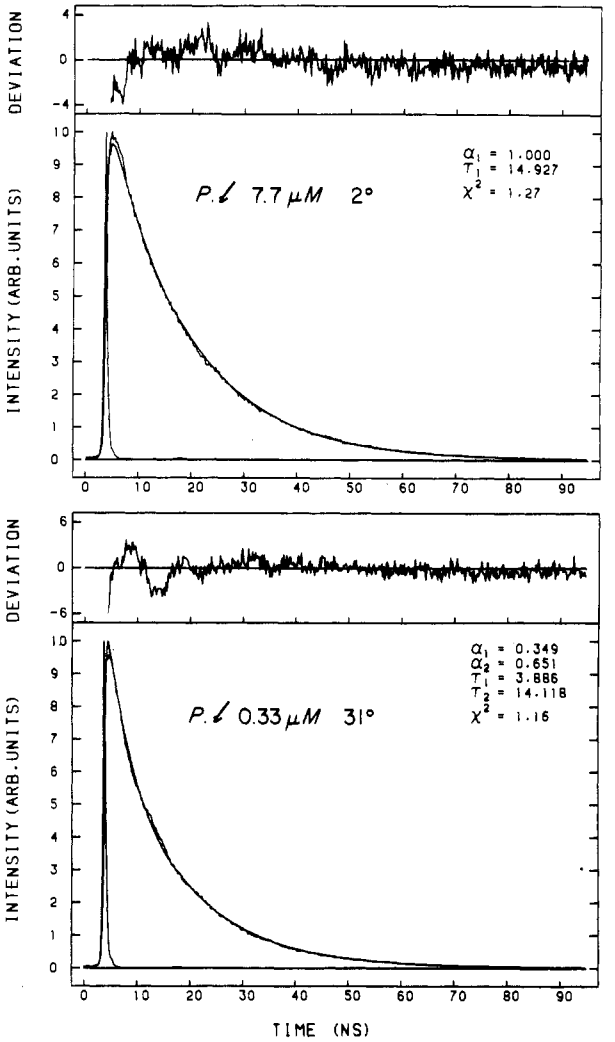


FIGURE 3: Fluorescence decay of *P.l.* LumP. Excitation was at 458 nm, and fluorescence was detected at 501 nm. (Upper panel) 7.7 $\mu$M, 2 °C; fitted function is $I = \exp[-t\ (\text{ns})/14.9]$. (Lower panel) 0.33 $\mu$M, 31 °C; $I = 0.35\exp(-t/3.9) + 0.65\exp(-t/14.1)$.

$I/I_f$ is the ratio of bound to free Lum fluorescence intensities, which varies from 0.93 to 1.45 for 40 °C to 0 °C for *P.l.* LumP. The results are listed in Table II. The $K_d$ is too small for reliable measurements to be made below 20 °C because the solutions have to be diluted below 10 nM and the fluorescence signal then is rather weak. By making a van't Hoff plot, we can extract $\Delta H° = 25$ kcal·mol$^{-1}$ and $\Delta S° = 50$ eu for the dissociation reaction. Table II also contains a remeasurement of *P.p.* LumP. The new preparation shows good agreement with the previous measurement of $p_b$, but the ligand is more tightly bound. Visser & Lee (1980) noted a variability of $K_d$ between their preparations of *P.p.* LumP and therefore only made a rough estimate of $K_d$. The recent preparations however do not show this variation, and a somewhat smaller and reproducible value for $K_d$ can now be concluded.

The fluorescence intensity of the Lum bound in *P.l.* LumP decays with time in a strictly exponential manner (Figure 3). This figure and the following figures of the same type show the narrow excitation pulse at about $t = 3$ ns, the fluorescence signal that is the slightly noisy line, and the fitted function that, in the case of the upper panel of Figure 3, is $I = \alpha_1 \exp[-t\ (\text{ns})/14.9]$. The deviations from the fit are shown in the top panel. The same single exponential decay of fluorescence was shown before for the *P.p.* LumP (Visser & Lee, 1980). The fluorescence lifetimes for the new preparations are the same

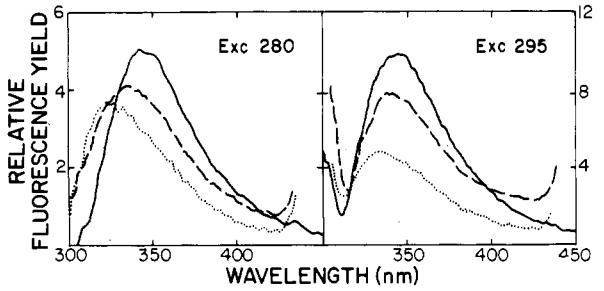


FIGURE 4: UV fluorescence spectra: L-tryptophan, 24 μM (—); *P.p.* LumP, 8.6 μM (– –); *P.l.* LumP, 8.8 μM (···); 22 °C. The Trp intensity is scaled down (4×, left; 2×, right) for comparison.

Table III: UV Fluorescence of Lumazine Proteins

| | Fluorescence Spectra excitation at 280 nm | | excitation at 295 nm | |
|---|---|---|---|---|
| | $\lambda_{max}$ (nm) | $\phi_F$[a] | $\lambda_{max}$ (nm) | $\phi_F$[a] |
| L-Trp | 348 | 0.20 | 347 | 0.20 |
| *P.p.* LumP | 339 | 0.08 | 342 | 0.11 |
| *P.l.* LumP | 328 | 0.06 | 337 | 0.06 |

| | Fluorescence Lifetimes[b] | | | | | |
|---|---|---|---|---|---|---|
| | concn (μM) | $\alpha_1$ | $\tau_1$ (ns) | $\alpha_2$ | $\tau_2$ (ns) | detection |
| *P.p.* LumP | 11 | 0.49 | 1.9 | 0.51 | 6.9 | broad band |
| | 20 | 0.41 | 2.0 | 0.59 | 6.7 | 335 nm |
| | 2 | 0.43 | 1.2 | 0.57 | 6.5 | 335 nm |
| *P.l.* LumP | 1 | 0.68 | 1.2 | 0.32 | 6.3 | broad band |
| | 10 | 0.63 | 1.7 | 0.37 | 6.3 | 335 nm |
| | 1 | 0.63 | 1.8 | 0.37 | 6.3 | 335 nm |

[a] 22 °C; the published $\phi_F$ for Trp is used as a standard (Teale & Weber, 1957). For the LumPs the $\phi_F$ is calculated on the absorbance of the protein part, excluding the Lum. [b] Excitation at 295 nm, 4 °C. Detection is broad band with a Schott filter WG320 cutting on at 320 nm plus a Balzers K36 band-pass filter or is with a monochromator set at 335 nm and 8-nm band-pass. Average lifetimes are as follows: *P.p.* LumP, 4.5 ns; *P.l.* LumP, 3.2 ns.

(ns, 2 °C): *P.p.* LumP, 14.4 ± 0.2 ($n$ = 10); *P.l.* LumP, 14.8 ± 0.4 ($n$ = 7).

The lower panel of Figure 3 is another demonstration of the dissociation equilibrium. The *P.l.* LumP is diluted 20× and is at 31 °C, and the fluorescence decay no longer is single exponential. It is fitted to a two-exponential function, $I = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$ (see legend). The shorter lifetime $\tau_1$ represents free Lum and the longer one $\tau_2$ the bound ligand. An increase in temperature shortens $\tau_2$ (e.g., to 11.9 ns at 40 °C), but at constant temperature it is otherwise unaffected by other conditions that favor dissociation, such as dilution or lower ionic strength.

When the excitation wavelength is less than 300 nm, two fluorescence bands are now observed. One is the same bound Lum fluorescence (Figure 2) since Lum absorbs below 300 nm (Figure 1), and the other is the 350-nm-region fluorescence originating from the Tyr and Trp residues of the protein. These UV fluorescence spectra are shown in Figure 4 along with the fluorescence of free Trp scaled down for convenient comparison with the LumP spectra. The rise in intensity at the long-wavelength end is the beginning of the bound Lum fluorescence.

The LumP spectra in Figure 4 have maxima that are blue shifted over that of free Trp, more so for the *P.l.* LumP. The numerical data are collected in Table III. The relative fluorescence yield on the Figure 4 ordinate is based on the total absorbance whereas in Table III the quantum yield $\phi_F$ is based on just the protein (Tyr + Trp) contribution to the absorbance, that is, with the Lum contribution subtracted. Separate ex-

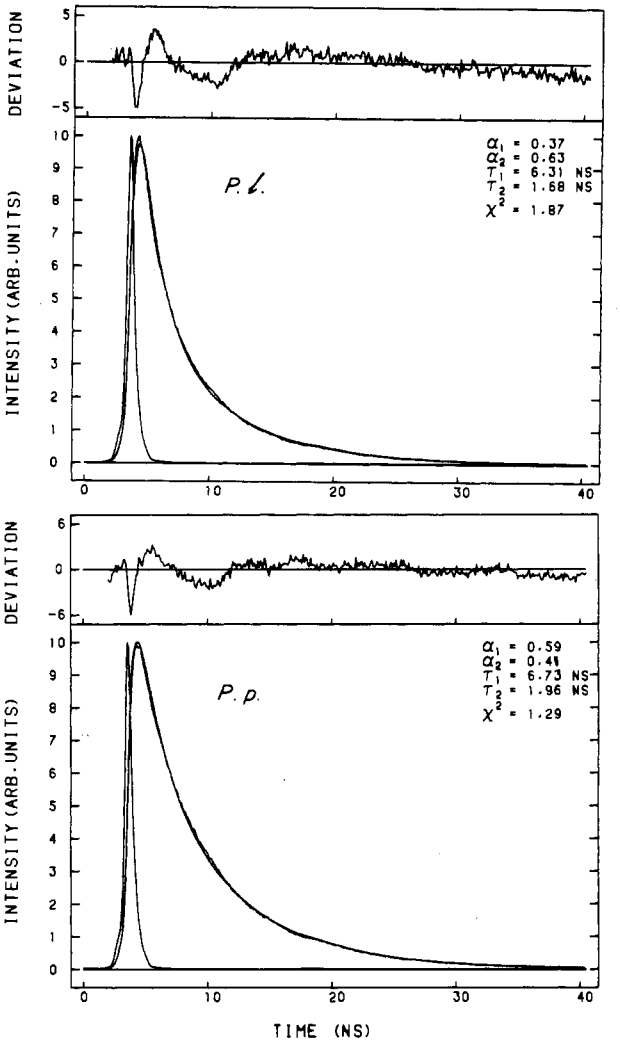


FIGURE 5: Time decay of UV fluorescence with a two-exponential fit. Excitation was at 295 nm, and fluorescence was at 335 nm; 4 °C. (Top panel) *P.l.* LumP, 10 μM. (Bottom panel) *P.p.* LumP, 20 μM. Numerical data are in Table III.

periments have shown that the absorption spectrum of the holoprotein is the sum of the separate components, Lum and P (O'Kane & Lee, 1985b). For 295-nm excitation, right panel, Figure 4, the *P.p.* LumP has a higher relative fluorescence yield (also $\phi_F$, see Table III) than for 280-nm excitation, left panel, Figure 4, but the spectral maximum is not changed. For *P.l.* LumP on the other hand, the 280- to 295-nm excitation change shifts the fluorescence maximum 9 nm to longer wavelength without any effect on the quantum yield. Fluorescence spectra were also measured at 2 °C, and very similar results were obtained.

Figure 5 shows the time decay of the UV fluorescence. For both LumPs the decay is clearly a double-exponential function. In Table III are the results obtained for a number of variations of experimental conditions. In all cases the same two lifetimes are observed for both LumPs, 1.7 and 6.5 ns. The difference between the two proteins is the predominance of the 6.5-ns-lifetime component for *P.p.* LumP (83%) as compared to the *P.l.* LumP (67%).

Figure 6 compares the circular dichroism of the free ligand Lum (long-dashed line) and the two LumPs. The free Lum has negative Cotton effects at 409 and 258 nm and positive ones at 325 and 285 nm. The longer wavelength band has an intensity of $-9.1 \times 10^3$ deg cm$^2$ dmol$^{-1}$ ($\Delta\epsilon$ = 3.1 M$^{-1}$ cm$^{-1}$). These results are in agreement with the literature: negative bands at 415 ($-8.4 \times 10^3$ deg cm$^2$ dmol$^{-1}$) and 262 nm and

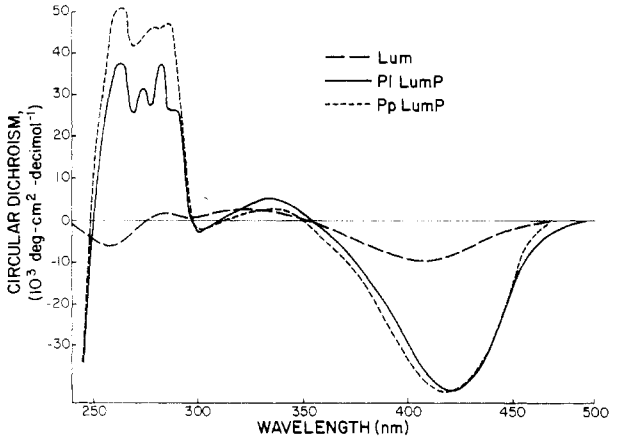


FIGURE 6: Circular dichroism of Lum (––), *P.p.* LumP (- - -), and *P.l.* LumP (—). All are at 22 °C, at pH 7, around 45 $\mu$M, and in standard buffer except that 2-mercaptoethanol is not present in the Lum solution.

positive bands at 330 and 285 nm (Harders et al., 1974). The bands correspond to the absorption maxima (Figure 1) except for the one at 325 nm, which according to the excitation results in Figure 1 belongs to the trace of the 7-oxo derivative. Both the LumPs have strongly intensified Cotton effects at the longest wavelength band, *P.p.* LumP at 418 nm and *P.l.* LumP at 421 nm, and of the same magnitude, $-41 \times 10^3$ deg cm$^2$ dmol$^{-1}$. Below 300 nm the Cotton effects are strongly positive and exhibit fine structure. Here the intensity is dominated by the Tyr and Trp residues' contribution.

The addition of KI efficiently quenches the fluorescence of Lum and LumP solutions. The data are not shown, but the steady-state fluorescence of free or bound Lum is reduced in a linear manner following the Stern–Volmer relationship $F_0/F = 1 + K[\mathrm{KI}]$, where $F_0$ is the fluorescence intensity without and $F$ with concentrations of KI up to 0.1 M and $K$ is the Stern–Volmer constant (2 °C, 50 $\mu$M): Lum, 57 M$^{-1}$; *P.p.* LumP, 21 M$^{-1}$; *P.l.* LumP, 12 M$^{-1}$. The KI did not affect the fluorescence spectral distribution. The quenching is largely diffusional since the decay rates fit the linear relationship $\tau^{-1} = \tau_0^{-1} + k_q[\mathrm{KI}]$, where $\tau$ and $\tau_0$ are the average lifetimes with and without KI and $k_q$ is the bimolecular quenching constant. When plotted (not shown), the data for the two LumPs fall on the same line, and from the slopes the values of $k_q$ are obtained ($10^9$ M$^{-1}$ s$^{-1}$): Lum, 4.3 (3 °C), 6.2 (20 °C); both LumPs, 0.86 (2 °C).

The 2-mercaptoethanol used in the buffer also exhibits a weak quenching ability for the free or bound Lum fluorescence, with Stern–Volmer constants in the same order as above for KI (20 °C): Lum, 17 M$^{-1}$; *P.p.* LumP, 4 M$^{-1}$; *P.l.* LumP, 1.5 M$^{-1}$. At the concentrations used here the 2-mercaptoethanol quenching of LumP fluorescence can be neglected. No quenching of the fluorescence of the Trp/Tyr residue ($K <$ 0.5 M$^{-1}$) could be observed.

Figure 7 shows the time decay of the emission anisotropy, measured for Lum bound in *P.l.* LumP in the top panel and for the Trp emission for the two LumPs in the lower two panels. It is clear that the anisotropy $A$ of the Lum in *P.l.* LumP relaxes in a closely first-order manner, $A = A_0 \exp(-t/\phi_0)$, where the relaxation time $\phi_0 = 17.4$ ns (2 °C). For the Trp emissions on the other hand, the decay is more complex and a biexponential function is required. For the *P.p.* LumP (bottom panel) the decay is almost wholly by a rapid process, $\phi_1 = 0.4$ ns, although there is a little contribution from a $\phi_2$ of 4.4 ns. A significant decay process however in the *P.l.* LumP (middle panel) is at a rate $\phi_2 = 15.5$ ns, close to that

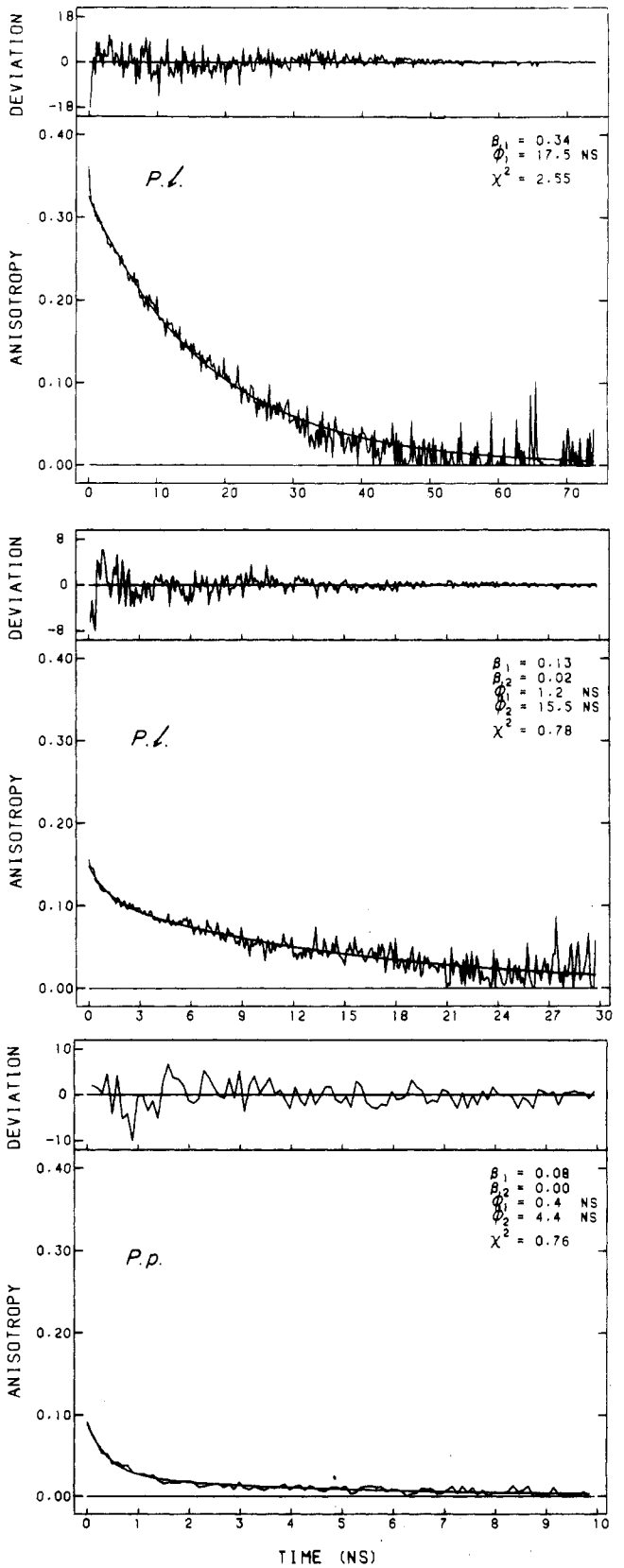


FIGURE 7: Time decay of emission anisotropy from bound Lum (top panel) or Trp residue (lower two panels). (Top panel) *P.l.* LumP, 7.7 $\mu$M, 2 °C, 458-nm excitation, 501-nm fluorescence. (Middle panel) *P.l.* LumP, 10 $\mu$M, 4 °C, 295-nm excitation, broad-band detection (see footnote *b*, Table III). (Bottom panel) *P.p.* LumP, 11 $\mu$M, 4 °C, 295-nm excitation, broad-band detection.

measured for the bound Lum ligand.

Observations of the anisotropy of bound Lum under various solution conditions are collected in Table IV. At 2 °C the

Table IV: Decay of Lum Emission Anisotropy[a]

| condition[b] | $T$ (°C) | concn ($\mu$M) | $A_0$ | $\phi_0$[c] (ns) |
|---|---|---|---|---|
| *P.p.* LumP | 2 | 20 | 0.349 | 19.9 |
| after 20 h at 22 °C | 22 | 20 | 0.315 | 11.4 |
| | 2 | 20 | 0.320 | 19.8 |
| KI, 25 mM | 2 | 20 | 0.314 | 20.2 |
| KI, 50 mM | 2 | 20 | 0.320 | 20.2 |
| KI, 100 mM | 2 | 20 | 0.323 | 20.7 |
| KI, 250 mM | 2 | 20 | 0.305 | 20.6 |
| KI, 250 mM | 20 | 20 | 0.280 | 14.4 |
| *P.l.* LumP | 2 | 602 | 0.320 | 17.8 |
| after 20 h at 22 °C | 22 | 0.33 | 0.24 | 11.1 |
| new soln, heated | 22 | 0.33 | 0.24 | 10.6 |
| and cooled in | 30 | 0.33 | 0.21 | 8.0 |
| sequence | 40 | 0.33 | 0.024 | 9.0 |
| | 10 | 0.33 | 0.21 | 15.3 |
| | 2 | 0.33 | 0.31 | 22.0 |
| new soln | 2 | 0.33 | 0.395 | 16.8 |
| Figure 7 | 2 | 7.7 | 0.337 | 17.5 |
| KCl, 500 mM | 2 | 7.7 | 0.340 | 16.4 |
| KI, 25 mM | 2 | 7.7 | 0.348 | 17.6 |
| KI, 50 mM | 2 | 7.7 | 0.320 | 20.2 |
| KI, 100 mM | 2 | 7.7 | 0.351 | 17.2 |
| KI, 250 mM | 2 | 7.7 | 0.317 | 20.5 |
| | 19 | 7.7 | 0.355 | 9.4 |
| | 4 | 10 | 0.342 | 17.4 |

[a] Fitted to the following equation: anisotropy $A = A_0 \exp(-t/\phi_0)$. [b] All in standard buffer. [c] Averages at 2 °C are as follows: *P.l.* LumP, 17.5 ns; *P.p.* LumP, 19.5 ns.

averages are as follows: *P.p.* LumP, $A_0 = 0.33$, $\phi_0 = 19.6$ ns; *P.l.* LumP, $A_0 = 0.36$, $\phi_0 = 17.3$ ns. The parameters are unchanged if the solution is kept overnight at room temperature. On the addition of KI the emission anisotropy of the unquenched fluorescence of *P.l.* LumP is not significantly affected, but for *P.p.* LumP there is a slight drop in the initial anisotropy, $A_0$. When the *P.l.* LumP solution is at 40 °C, the initial anisotropy drops to almost zero (data were not deconvoluted), consistent with the dissociation of Lum as concluded from the fluorescence change in Figure 2. When the solution is returned to 2 °C and after the 3-h incubation, the original parameters are completely restored, again proof of total reversibility as in Figure 2. There is also no change in the parameters for *P.l.* LumP when its concentration at 2 °C changes from 0.33 to 602 $\mu$M.

## Discussion

The literature abounds with examples of the use of the fluorescence properties of a molecule to gain information about its environment within a protein (Weber, 1976; Lakowicz, 1983; Steiner, 1983). For LumP the fluorescence maximum of the ligand, which is 490 nm in free solution, shifts when it binds to the protein to 475 nm. The binding also causes an increase in $\phi_F$ and $\tau$, and all these changes are the same for both types of LumP. The high value of the $A_0$ and the correspondence of the $\phi_0$ for bound Lum to the protein's rotational correlation time mean that the ligand is rigidly attached to the protein, slightly more rigidly in the case of *P.l.* LumP ($A_0 = 0.36$) than in *P.p.* LumP ($A_0 = 0.33$). Therefore, the blue shift and other changes in the fluorescence of Lum are accounted for by its immobilization in the protein binding site; the same changes are seen by dissolving lumazines in a rigid solution (Sun et al., 1972). The polarity of the medium also has an effect on the fluorescence of Lum; in ethanol the maximum is red shifted about 5 nm over water. Therefore, it is concluded that if there were any such difference in effective polarity or hydrophobicity, as between water and ethanol, between the binding sites in the two proteins, identical fluorescence properties would not be observed.

The first major result of this present work is that the visible fluorescence properties of the two LumPs are identical. They have the same property of strictly exponential decay of the fluorescence intensity, unusual for protein-bound fluorophores.

The spectral data also suggest that the lumazine ring is probably exposed to solvent rather than buried in the protein. To achieve the high $\phi_F$, it would be of advantage for the electronic ring system not to be in contact with fluorescence quenchers like Trp, Tyr, Cys, or His residues. This requirement would be fulfilled and other properties of the Lum accounted for if Lum attached itself by burying the ribityl group into the protein, with the lumazine ring lying flat on the surface. An argument can be made for this idea from the circular dichroism in Figure 6. The intensity of the visible band is a measure of the asymmetry of the environment of the Lum, and this intensity is much increased in LumP. Harders et al. (1976) suggest a factor contributing to this intensity is the interaction of the 2′-hydroxyl of the ribityl side chain with the lumazine ring. The above model of the binding site implies a very asymmetric environment for the bound ligand. With flavodoxin for comparison the corresponding longest wavelength band of its ligand FMN has a circular dichroism only about half as intense as LumP (Edmondson & Tollin, 1971). In flavodoxin the FMN is somewhat buried in the protein, so its surroundings would be more uniform than for the lumazine ring in LumP if one side was protein and the other solvent water.

The final piece of evidence for a surface binding site is that the Raman vibrational frequencies are the same between free Lum and both LumPs (Vervoort et al., 1983). Binding within the protein would be expected to alter hydrogen bonding to the lumazine ring atoms and thus affect their vibrational frequencies (Irwin et al., 1980; Visser et al., 1983; Müller et al., 1983). The steady-state Stern–Volmer and bimolecular quenching constants are also consistent with ready accessibility of the lumazine ring to approach by $I^-$.

Another important property of the attached Lum is that its anisotropy decays as a single exponential process at a rate expected for the rotational relaxation of the protein as a whole (Figure 7, Table III; for calculations see Visser & Lee, 1980). The lumazine ring therefore must maintain a fixed orientation with respect to the protein since it displays no "floppiness", which would be indicated by a faster relaxation time and which is indeed observed for the fluorescence from the Trp residue (Figure 7).

Both LumPs are affected by changes in solution properties in a parallel manner, and the effects can be interpreted unambiguously in both cases as arising from the dissociative equilibrium. The basis for the interpretation has been discussed before for the case of *P.p.* LumP, and it is largely a consequence of the ideality of its fluorescence behavior. On dilution, increase in temperature, decrease in ionic strength, or shift to pH extremes, the LumP visible fluorescence red shifts, a second shorter lifetime appears in the decay, and the polarization falls; all these are due to the increase in concentration of free Lum (Visser & Lee, 1980). At 31 °C and 0.3 $\mu$M *P.l.* LumP, there is about a 50% effect on all three of these parameters. Therefore, we can estimate the $K_d \sim 0.3$ $\mu$M (50 mM $P_i$) at this temperature.

The important difference then between *P.p.* LumP and *P.l.* LumP is a factor of about 10 times tighter binding for *P.l.* LumP (e.g., Table II, for 20 °C). In fact, below 20 °C the binding in *P.l.* LumP is too tight to measure by these fluorescence techniques, which was not the case for *P.p.* LumP. Since the $K_d$'s are below 1 $\mu$M for most experiments, the

concentrations of LumPs have to be below this for both free and bound to be present, and the resulting signal intensities in fluorescence polarization are low and limit the precision with which measurements can be made.

If we take the environment of the lumazine ring to be nearly the same between the two proteins, then we cannot explain this 10 times difference in association as coming from a stronger interaction between the ring system and the binding site. The temperature dependence of $K_d$ results in the same $\Delta H^\circ \sim 25$ kcal·mol$^{-1}$ for both LumPs. The difference in binding therefore is a consequence of an entropic effect or other non-temperature-dependent interaction, e.g., electrostatic; about 10 kcal·mol$^{-1}$ is involved.

The amino acid composition of the two LumPs is not the same (O'Kane et al., 1985). They both contain one Trp residue, but the *P.l.* LumP has a higher content of hydrophobic amino acids, and it also displays a more acidic behavior on ion-exchange chromatography and a pH optimum for fluorescence 0.5 unit lower than for *P.p.* LumP. They both have exactly the same anhydrous molecular weights, so the difference in relaxation time $\phi_0$ (Table IV) means either that *P.l.* LumP has a lower degree of hydration $h$ ($\phi_0 = M_r \eta(\bar{v} + h)/NkT$; Visser & Lee, 1980) or that it deviates from a spherical shape (O'Kane & Lee, 1985a).

Secondary-structure differences between the two LumPs are suggested by both the UV circular dichroism and the Trp fluorescence behavior. In the aromatic amino acid region the two LumPs have a strong positive Cotton effect, 50% more intense for *P.p.* LumP than *P.l.* LumP (Figure 6). A more asymmetric environment is therefore implied for the Tyr and Trp residues in *P.p.* LumP. Taking the circular dichroism further into the UV to look at secondary structure in more detail would be desirable but is precluded by the absorption of 2-mercaptoethanol in the buffer, required for stabilization. Therefore, we have concentrated on comparing the Tyr/Trp fluorescence properties to show at least that the secondary structures are different. Both proteins give a UV fluorescence arising mostly from Trp (Figure 4), blue shifted over Trp in water, and this shift is usually taken to mean that the Trp is buried in the protein structure in a hydrophobic environment. The further shift to shorter wavelength of *P.l.* LumP on 280-nm excitation (Figure 4, left panel) is probably due to Tyr contribution to the total fluorescence envelope. At 295-nm excitation (right panel) where Tyr does not contribute, the fluorescence spectra of the proteins are still different. Therefore, even though the Tyr/Trp ratio is higher for *P.p.* LumP (3:1) than for *P.l.* LumP (2:1; O'Kane & Lee, 1985a), for *P.p.* LumP the Tyr does not contribute to the fluorescence emission since the fluorescence yield increases on going from 280 to 295 nm (Table III). This is in accordance with the average lifetime for the UV fluorescence, which is longer for *P.p.* LumP than for *P.l.* LumP (Table III).

The excitation spectra of Figure 1 suggest that there is some energy transfer from Trp or Tyr to bound Lum, since the intensity at 280 nm is slightly higher than would be predicted from the bound Lum transmission spectrum. There is more energy transfer in the case of *P.l.* LumP, but in either protein it is not much, probably because the overlap of fluorescence and Lum absorbance is small. In free solution Lum is subject to photochemical decomposition, and this is probably more efficient in the UV region, accounting for the intensity deficiency of its excitation spectrum over its transmission. Lum bound to the protein is more stable.

The Trp fluorescence decay of both proteins is nonexponential as is observed with other proteins (Munro et al., 1979). A minimum decay model is that of a sum of two exponential functions, and the time constants might reflect different Trp conformations (Szabo & Rayner, 1980). The values of the two lifetime components are the same for both proteins; only their relative weight is reversed. Their time-resolved anisotropy decay is also complex, in contrast to the bound Lum (Figure 7), and the presence of a fast relaxation time, $\sim 1$ ns, in both proteins suggests rapid motion of the Trp residue and that its conformation interconverts on a (sub)nanosecond time scale. Both proteins differ significantly in this respect. *P.p.* LumP Trp depolarizes almost completely in $<1$ ns, while *P.l.* LumP Trp relaxes slightly more slowly, in $\sim 1$ ns, but also with a contribution rotating with the protein as a whole, $\phi \sim 16$ ns. In terms of a "wobble in cone" model (Lipari & Szabo, 1980) the conclusion is that in *P.p.* LumP the cone semiangle has a limiting value of 55° (complete depolarization). In *P.l.* LumP the motion is much more restricted with smaller angles involved.

The bioluminescence in the cell involves LumP and a second protein, luciferase ($M_r$ 80 000). Chemical energy generated by reaction of $FMNH_2$, $O_2$, and an aliphatic aldehyde, catalyzed by the luciferase (E), is transformed into electronic excitation of the bound Lum. At least 63 kcal·mol$^{-1}$ is required as a minimum for the excitation (0–0′ transition), and the energy needs to be transformed adiabatically and with maximum efficiency (Lee, 1968). Proximity of the site of energy generation and the Lum acceptor would seem to be a necessary factor to satisfy these conditions, and in fact, complexation between the luciferase and LumP has been described (Visser & Lee, 1982). The topography of this protein–protein complex has not been studied, but one can imagine that the complex should have a specific structure designed to achieve a certain definite alignment of the lumazine ring with respect to some dimension of the protein–protein system.

The mechanism of chemical to electronic energy conversion is not known, but if long-range electronic energy transfer is taking place, then correct orientation of the lumazine ring with respect to the donor is a critical factor (Dale & Eisinger, 1975). If the mechanism on the other hand is one requiring orbital overlap, e.g., electron exchange, then both orientation and proximity to the source of chemical energy would have to be under control. The two LumPs therefore have designed themselves to restrict the motion of the Lum to correspond to that of the whole macromolecule. Thus if the E–P structure is specified by certain protein–protein interactions, then the Lum as a result will be placed in its correct position.

The bioluminescent bacteria are often symbiotically associated with a fish, the light serving a biological function for the host. The spectral distribution of the light therefore must be an additional requirement that the LumP serves, as well as maximizing the efficiency at which it is generated. The apoprotein achieves this by providing a certain binding site environment for the bound ligand, which is the same for both LumPs.

The interesting question that now arises is how the two LumPs have solved the same functional problem under different environmental pressure, primarily that of ambient temperature. The two LumPs are analogous to isozymes—different proteins but with the same "enzymatic" (i.e., light-emission) function. *P.l.* LumP had to evolve a secondary structure that binds the ligand to a degree that they remain together at the temperature of warm ocean water. The dissociation probably serves a function too in connecting the Lum to the metabolic pool. Presumably both LumPs evolved from a common ancestral protein, but they must have preserved

certain parts of the primary sequence to provide a binding site that gives the Lum its required spectral properties and rigid orientation. The molecular strategy that has been used by those two LumPs is probably a rather subtle one that will probably not be evident until the primary sequence and good structural models are determined.

ACKNOWLEDGMENTS

We thank T. Ykema for help with some of the data analysis.

**Registry No.** Lum, 5118-16-1.

REFERENCES

Chien, Y., & Weber, G. (1973) *Biochem. Biophys. Res. Commun. 50*, 538–543.

Dale, R. E., & Eisinger, J. (1975) in *Concepts in Biochemical Fluorescence* (Chen, R. F., & Edelhoch, H., Eds.) pp 115–184, Marcel Dekker, New York.

Edmondson, D. E., & Tollin, G. (1971) *Biochemistry 10*, 113–124.

FitzGerald, J. (1978) Ph.D. Thesis, Monash University, Melbourne, Australia.

FitzGerald, J., & Lee, J. (1978) in *Microbial Ecology* (Loutit, M. W., & Miles, J. A. R., Eds.) pp 40–41, Springer-Verlag, West Berlin.

Gast, R., & Lee, J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 833–837.

Grinvald, A., & Steinberg, I. Z. (1974) *Anal. Biochem. 59*, 583–598.

Harders, H., Forster, S., Voelter, W., & Bacher, A. (1974) *Biochemistry 13*, 3360–3364.

Irwin, R. M., Visser, A. J. W. G., Lee, J., & Carreira, L. A. (1980) *Biochemistry 19*, 4639–4646.

Koka, P., & Lee, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3068–3072.

Koka, P., & Lee, J. (1981) *Proc. Annu. Meet. Am. Soc. Photobiol., 9th 9*, 192.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum, New York.

Lee, J. (1968) in *Energetics and Mechanism in Radiation Biology* (Phillips, G. O., Ed.) pp 269–275, Academic Press, London.

Lee, J. (1982) *Photochem. Photobiol. 36*, 689–697.

Lee, J., & Koka, P. (1978) *Methods Enzymol. 57*, 226–234.

Lee, J., Carreira, L. A., Gast, R., Irwin, R. M., Koka, P., Small, E. D., & Visser, A. J. W. G. (1981) in *Bioluminescence and Chemiluminescence* (DeLuca, M., & McElroy, W. D., Eds.) pp 103–112, Academic Press, New York.

Lipari, G., & Szabo, A. (1980) *Biophys. J. 30*, 489–506.

Müller, F., Vervoort, J., Lee, J., Horowitz, M., & Carreira, L. A. (1983) *J. Raman Spectrosc. 14*, 106–117.

Munro, I., Pecht, I., & Stryer, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 56–60.

O'Kane, D. J., & Lee, J. (1985a) *Biochemistry* (second of five papers in this issue).

O'Kane, D. J., & Lee, J. (1985b) *Biochemistry* (fourth of five papers in this issue).

O'Kane, D. J., Karle, V. A., & Lee, J. (1985) *Biochemistry* (first of five papers in this issue).

Parker, C. A. (1968) *Photoluminescence of Solutions*, Elsevier, Amsterdam.

Small, E. D., Koka, P., & Lee, J. (1980) *J. Biol. Chem. 255*, 8804–8810.

Steiner, R. F., Ed. (1983) *Excited States of Biopolymers*, Plenum Press, New York.

Sun, M., Moore, T. A., & Song, P.-S. (1972) *J. Am. Chem. Soc. 94*, 1730–1740.

Szabo, A. G., & Rayner, D. M. (1980) *J. Am. Chem. Soc. 102*, 554–563.

Teale, F. W. J., & Weber, G. (1957) *Biochem. J. 65*, 476–482.

van Hoek, A., Vervoort, J., & Visser, A. J. W. G. (1983) *J. Biochem. Biophys. Methods 7*, 243–254.

Vervoort, J., O'Kane, D. J., Carreira, L. A., & Lee, J. (1983) *Photochem. Photobiol. 37*, 117–119.

Visser, A. J. W. G., & van Hoek, A. (1979) *J. Biochem. Biophys. Methods 1*, 195–208.

Visser, A. J. W. G., & Lee, J. (1980) *Biochemistry 19*, 4366–4372.

Visser, A. J. W. G., & Santema, J. S. (1981) *Photobiochem. Photobiophys. 3*, 125–133.

Visser, A. J. W. G., & Lee, J. (1982) *Biochemistry 21*, 2218–2226.

Visser, A. J. W. G., Vervoort, J., O'Kane, D. J., Lee, J., & Carreira, L. A. (1983) *Eur. J. Biochem. 131*, 639–645.

Visser, A. J. W. G., Ykema, T., van Hoek, A., O'Kane, D. J., & Lee, J. (1985) *Biochemistry* (fifth of five papers in this issue).

Wampler, J. E. (1978) in *Bioluminescence in Action* (Herring, P. J., Ed.) pp 1–48, Academic Press, London.

Weber, G. (1976) in *Excited States of Biological Molecules* (Birks, J. B., Ed.) pp 363–374, Wiley, New York.