Purification of Lumazine Proteins from *Photobacterium leiognathi* and *Photobacterium phosphoreum*: Bioluminescence Properties†

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ABSTRACT: Bright strains of the marine bioluminescent bacterium *Photobacterium leiognathi* produce a "lumazine protein" in amounts comparable to that previously found in *Photobacterium phosphoreum*. New protocols are developed for the purification to homogeneity of the proteins from both species in yields up to 60%. In dimmer strains the amounts of lumazine protein in extracts are less, and also there is an accompanying shift of the bioluminescence spectral maximum to longer wavelength, 492 nm. Both types of lumazine proteins have identical fluorescence spectra, with maxima at 475 nm, so it is suggested that, whereas lumazine protein is the major emitter in bright strains, there is a second emitter also present with a fluorescence maximum at longer wavelength. The two species of lumazine protein have the same 276 nm/visible absorbance ratio, 2.2, but differ in visible maxima: *P. phosphoreum*, 417 nm; *P. leiognathi*, 420 nm. For the latter the bound lumazine has $\epsilon_{420} = 10\,100\ M^{-1}\ cm^{-1}$, practically the same as in free solution. The two lumazine proteins also differ quantitatively in their effect on the in vitro bioluminescence reaction, i.e., at blue shifting the bioluminescence spectrum or altering the kinetics. The *P. phosphoreum* lumazine protein is more effective with its homologous luciferase or with *P. leiognathi* luciferase than is the lumazine protein from *P. leiognathi*. These differences may have an electrostatic origin.

The bioluminescent bacteria emit light in a broad spectral distribution with a spectral maximum in the range 472–505 nm (Spruit-van der Burg, 1950; Seliger & Morton, 1968; Cline & Hastings, 1974; Gast & Lee, 1978) except for one type that has a bimodal emission, maxima at 545 nm and around 490 nm (Ruby & Nealson, 1978). Several genera of luminous bacteria are known, but the most commonly occurring are *Vibrio* and *Photobacterium* (Baumann et al., 1984; Baumann & Baumann, 1984). Although the type dependence of the bioluminescence has not received systematic study hitherto, a survey of the literature shows that identified *Photobacterium* types mostly have spectral maxima at the blue end of the range, while *Vibrio* types have maxima at longer wavelengths than 482 nm.

It is the experience of this laboratory, and this generalization may also be gleaned from the literature, that the amount of bioluminescence from the cell also depends on cell type (Lee et al., 1974; Lee & Koka, 1978; Karl & Nealson, 1980; Lee, 1982). A reasonably reproducible (±25%) index of this "bioluminescence potential" is the maximum intensity of bioluminescence that can be obtained in liquid culture. For most types it is well-known that regular subculturing on solid agar is essential for maintaining a high light emission property of a strain (Nealson, 1978). Once this is done, the maximum bioluminescence potential is in the order *Photobacterium* > *Vibrio fischeri* > *Vibrio harveyi*. These are the most commonly studied types, and in this laboratory we find a consistent difference of over 30-fold between the brightest *Photobacterium* types and *V. harveyi* strains.

What determines the maximum bioluminescence potential and the spectral distribution in each type? Certainly one factor determining the potential is the amount and activity of its bacterial luciferase [M_r ~80 000; for a recent review see Ziegler & Baldwin (1981)]. However, although some dim strains do have depleted levels of luciferase, others do not, and the amounts and optimal bioluminescence activities of luciferase in the three classes above appear to be about the same (Hastings et al., 1978).

Gast & Lee (1978) identified a second protein involved in the bioluminescence of a bright strain A13 of *Photobacterium phosphoreum*. The protein was called "lumazine protein" (LumP)[1] because it had a highly fluorescent ligand characterized by Koka & Lee (1979) as 6,7-dimethyl-8-ribityllumazine. LumP was concluded to be the bioluminescence emitter in this strain because its fluorescence was identical with the bioluminescence spectrum, it was produced in large quantity, and when included in the in vitro reaction, it stimulated the activity of the *P. phosphoreum* luciferase as well as changed its bioluminescence spectrum [Gast & Lee, 1978; Lee & Koka, 1978; Koka & Lee, 1979; for a review see Lee et al. (1981)].

Koka & Lee (1979) speculated that LumP might be ubiquitous among the luminous bacteria and that their different bioluminescence spectra resulted from the type dependence of the lumazine protein's fluorescence distribution. Indeed LumPs have now been found in a second strain of *P. phosphoreum*, NCMB844 (Lee, 1982), and in two strains of *Photobacterium leiognathi* (Lee, 1982; Vervoort et al., 1983). In the 545-nm bioluminescence strain of *V. fischeri*, a yellow-fluorescent protein has been isolated, and it appears to be functionally similar to the LumP in *P. phosphoreum* (Leisman & Nealson, 1982).

In this report we describe detailed protocols for purification in high yields of LumPs from three types of *Photobacterium*. The preparations are spectrally homogeneous and have iden-

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[1] Abbreviations: NCMB, National Collection of Marine Bacteria; ATCC, American Type Culture Collection; PMSF, phenylmethanesulfonyl fluoride; LumP, lumazine protein; 2-ME, 2-mercaptoethanol; F_{470}, arbitrary fluorescence intensity at 470 nm with excitation at 420 nm; F_{520}, arbitrary fluorescence intensity at 520 nm with excitation at 470 nm; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FMN, riboflavin 5′-phosphate; SDS, sodium dodecyl sulfate.

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Table I: Purification of Lumazine Protein from *P. leiognathi* Strain A2D[a]

step	A_{280}	A_{420}	A_{280}/A_{420}	F_{470}	F_{520}	F_{470}/F_{520}	luciferase (10^{12} photons·s^{-1})
(1) cell-free extract[b]	25540	766	33	2516	613	4	5.1×10^6
(2) DEAE Sepharose: step elution	6330	257	25	2334	220	11	5.6×10^5
(3) gel filtration	268	84	3.2	1762	129	14	1.4×10^3
(4) DEAE-Sepharose: gradient elution							
applied to step 5	115	40	2.9	1050	99	11	ND[d]
pooled	66	30	2.2	642	39	16	ND
(5) gel filtration, AcA-54[c]	90	42	2.2	870	46	19	ND
final pool	153	70	2.2	1510	85	18	ND
% recovery	0.6	9		60	14		$<10^{-7}$

[a] All procedures were at 5 °C in standard buffer. A = absorbance × total volume (mL); F = fluorescence intensity × total volume, in arbitrary units referenced to a sodium fluorescein standard; F_{470} = 420 → 470 nm; F_{520} = 470 → 520 nm (excitation → fluorescence). The purifications are standardized to 1 kg wet weight of starting materials. Luciferase units are bioluminescence activity per milliliter × total volume (mL). [b] Turbidity in the cell extract prevents an accurate measurement of fluorescence and absorbance. Therefore, the values in this row are estimated by summing all fractions from step 2. [c] Combined pools of three columns. [d] ND, not detectable.

tical fluorescence properties but differ quantitatively in their properties in the bioluminescence reaction.

Materials and Methods

Source of Cells. *P. phosphoreum* strain A13 was from J. Fitzgerald (Swinburne Institute of Technology, Melbourne, Australia), NCMB844 was from E. Meighen, 11040 was from American Type Culture Collection (ATCC), and B402, B404, B408, B413, B415, B417, and B419 were from P. Baumann's collection (Reichelt & Baumann, 1973). *P. leiognathi* strains S1 and ECMG were from S. Ulitzur, 27561 was from ATCC, and B470, B471, B472, B475, and B477 were from P. Baumann. Type A2D was a brighter clone occurring spontaneously from S1. *V. fischeri* strain 7744 was from ATCC, and *V. harveyi* strain 392 (MAV), from J. W. Hastings.

Growth of Cells. For cell culture bright clones were first selected by plating. The *P. phosphoreum* was grown as previously described (Lee & Koka, 1978) while for *P. leiognathi* we used the following conditions (400 L): aeration, 340 L·min^{-1}; agitation, 300 rpm; 27 °C; pH 7.2. Harvesting the cells (cooled Sharples centrifuge) was started prior to the cells' reaching maximum bioluminescence since the centrifugation took 4–5 h. *P. leiognathi* strain S1 was harvested when the cell density was 160 Klett units (100 Klett units ≡ A_{660} = 2 ≡ 4×10^8 cells·mL^{-1}) and when the bioluminescence intensity was 1.2×10^{13} photons·s^{-1}·mL^{-1}. The strain A2D grew to higher cell densities and light output and was harvested at 220 Klett units and 2.5×10^{13} photons·s^{-1}·mL^{-1}. Approximately 0.5 h after starting the harvest the fermenter was chilled to 4 °C. The yields of wet cell paste from 400 L were as follows: S1, 1.2–1.8 kg; A2D, 2.0–2.6 kg; A13, 1.0–1.5 kg.

Luciferases. *V. fischeri* and *V. harveyi* luciferases were prepared by Drs. S. Reddigari and M. Ahmad using previously published procedures (Lee, 1982) with an additional step of chromatography on diphenylpropylamine–Sepharose (Holzman & Baldwin, 1982). The *P. phosphoreum* and *P. leiognathi* luciferases were produced as side products of the LumP preparations [Table I; see also supplementary material, Tables IV and V (see paragraph at end of paper regarding supplementary material)] and were purified to very high activities by procedures modified from Lee (1982) that will be published elsewhere. Luciferases were assayed for bioluminescence specific activity as described (Lee, 1972, 1982), at room temperature and with reduced riboflavin 5′-phosphate ($FMNH_2$) and aldehyde as indicated (photons·s^{-1}·A_{280}^{-1}): *V. fischeri*, 1.5×10^{14} (dodecanal); *V. harveyi*, 6×10^{13} (decanal); *P. phosphoreum*, 5×10^{14} (tetradecanal); *P. leiognathi*, 1.2×10^{15} (tetradecanal). All except *V. harveyi* showed 80–90% kinetic activity according to the method of Matheson & Lee (1983) (I. Matheson, unpublished observation).

Photometer calibration was made by reference to the NBS standard lamp and NBS absolute photodiode photometer via the luminol chemiluminescence reactions (Lee & Seliger, 1965; Lee et al., 1966; Matheson et al., 1984). Attention is drawn to the fact that other published values of luciferase bioluminescence activities (Hastings et al., 1978) or bioluminescence intensities in vivo (Karl & Nealson, 1980) are about 3 times higher than would be obtained by using the luminol calibration procedure [for a review of photometer calibration see Wampler (1978)].

Spectroscopy. Absorption, fluorescence, and bioluminescence spectra were obtained as previously described (Lee, 1982). Technical emission spectra were corrected by reference to the absolute fluorescence spectral distribution of a standard solution of quinine sulfate (Velapoldi & Mielenz, 1980). Self-absorption corrections were applied where necessary and were minimized by using cuvettes that had a path length of only 2 or 3 mm in the emission direction. Empirical analysis of the spectra was made by the method of reduced moments (Mulkerrin & Wampler, 1982). The generalized reduced moment is defined as

$$U_n = [\sum_i (\lambda_i - \bar{\lambda}) F_i] / \sum_i F_i \quad (1)$$

where F_i is the emission intensity at wavelength λ_i and $\bar{\lambda}$ is the spectral mean:

$$\bar{\lambda} = [\sum_i \lambda_i F_i] / \sum_i F_i \quad (2)$$

The derived parameters are

$$\text{skewness} = U_3 / U_2^{3/2} \quad (3)$$

$$\text{kurtosis} = (U_4 / U_2^2) - 3 \quad (4)$$

On this definition a Gaussian distribution will have zero kurtosis; a positive value means a sharper peak and a negative a flatter spectrum than Gaussian.

Results

Purification and Homogeneity of Lumazine Proteins. The LumPs from each strain of bacteria were obtained in 30–60% yields on the basis of the fluorescence at 470 nm (F_{470}) of the original cell-free extract (see supplementary material for details of the individual purifications). Table I summarizes the purification of LumP from *P. leiognathi* A2D. Luciferase and LumP overlap in their elution from DEAE-Sepharose (step 2) but can be quantitatively separated by preparative gel filtration on Sephadex G-75 (step 3) in the presence of CHAPS (0.25% residual activity). The pooled peak of LumP

Table II: Moment-Derived Spectral Shape Parameters

type	LumP (μM)	spectral max (nm)	spectral mean (nm)	skewness	kurtosis	max bioluminescence (10^{12} photons·s^{-1}·mL^{-1})
			Lumazine Protein Fluorescence			
P. phosphoreum A13		474	495	0.71	0.17	
P. leiognathi A2D		475	496	0.69	0.16	
P. leiognathi S1		475	496	0.72	0.14	
P. leiognathi B477		476	501	0.65	−0.23	
			In Vivo Bioluminescence[a]			
P. phosphoreum[b] A13		475	497	0.66	0.00	4
P. leiognathi A2D		477	499	0.61	−0.06	40
P. phosphoreum bright[c]		478	499	0.63	−0.01	2[d]
P. leiognathi S1		481	499	0.58	−0.14	20
P. sp. intermediate[e]		482	501	0.54	−0.16	1.5[f]
P. leiognathi B477		483	501	0.54	−0.20	2
P. leiognathi dim[g]		491	508	0.28	−0.56	0.01[h]
V. fischeri 7744		490	505	0.35	−0.46	2
			In Vitro Bioluminescence[i]			
P. leiognathi	1	490	505	0.42	−0.37	
	10	483	501	0.54	−0.19	
	30	481	500	0.58	−0.12	

[a] All spectra taken at room temperature, except for *P. phosphoreum* A13. [b] Spectrum taken at 12 °C. [c] Average of strains NCMB844, B402, B406, B408, B413, and B417. [d] NCMB844. [e] Average of *P. leiognathi* ATCC 27561 and ECMG and *P. phosphoreum* ATCC 11040, B404, B415, and B419. [f] ATCC 27561. [g] Average of strains B470, B471, B472, and B475; range of spectral maxima, 487–494 nm. [h] B470. [i] All with S1-type luciferase (10 μM), $FMNH_2$ (10 μM), tetradecanal (~50 μM), and S1 lumazine protein at the concentration indicated and at 2 °C.

eluted from DEAE-Sepharose (step 4) was virtually pure as judged by spectral parameters (A_{280}/A_{420} = 2.19) and represented 36% of the total LumP applied. The tail fractions were further purified by gel filtration on AcA-54. The overall recovery of LumP was approximately 60% with a recovery of 0.6% of the A_{280}. The recovery of LumP from *P. phosphoreum* A13 and *P. leiognathi* S1 was respectively 37 and 30%.

Homogeneity. The goal of these purification procedures is to obtain preparations that have both spectral and protein homogeneity. Spectral purity is judged by a minimum UV/visible absorbance ratio (A_{280}/A_{420} = 2.18 for pure material) and a maximum F_{470}/F_{520}. The purest fractions of *P. leiognathi* LumP show a contamination by flavoproteins equivalent to 0.1 mol % FMN (not shown). The LumPs are not contaminated by extraneous proteins because the fluorescamine-derivatized LumPs from the final stages of purification (data not shown) show only single bands on SDS–polyacrylamide gels with the exception of a low molecular weight amine contaminant that migrates with the dye front (Ragland et al., 1975). The level of detectability of fluorescamine-derivatized proteins is in the range from 10 to 100 μg. Consequently, the LumPs appear to be >97% pure on the basis of protein.

Absorption and Fluorescence Spectra. Figure 1 (top) shows that the absorption spectra of the LumPs purified from *P. phosphoreum* A13 and *P. leiognathi* A2D are similar but not identical. In the UV their two absorbance maxima are at the same wavelengths, 262 and 278 nm, and they have the same absorbance ratio of 278 nm/visible maximum, 2.2 [compared to 2.4–2.6 previously; Small et al. (1980)]. Their visible band maxima are slightly different: *P. phosphoreum*, 417 nm; *P. leiognathi*, 420 nm. The visible band of S1 is identical with that A2D, but the former has not been purified to minimum absorbance ratio (due to loss of the lumazine ligand during purification).

In 6 M guanidine hydrochloride the LumP as expected is denatured, and the visible absorption band is the same as for the authentic lumazine derivative, a maximum at 412 nm (Figure 1, bottom). In aqueous solution at pH 7, 6,7-dimethyl-8-ribityllumazine has ϵ_{407} = 10 300 M^{-1} cm^{-1} (Maley & Plaut, 1959), so the extinction coefficient of the protein-bound ligand (Figure 1, top) can be calculated by substitution:

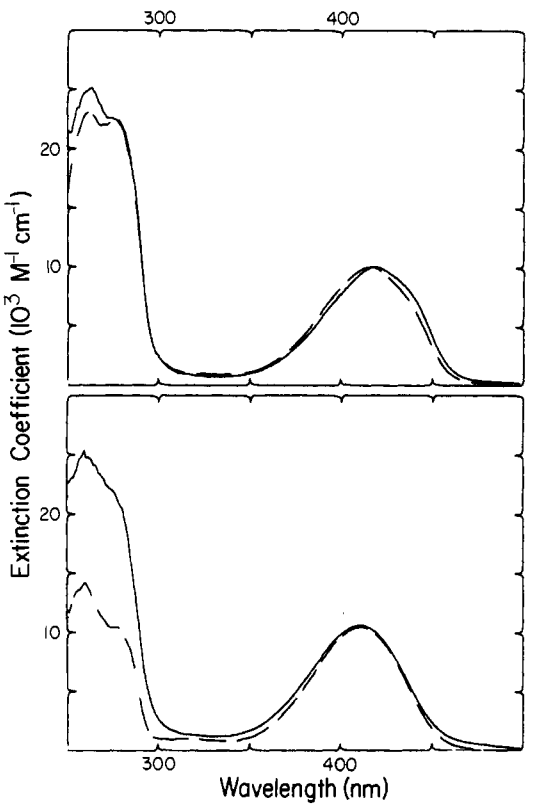

FIGURE 1: (Top) Absorption spectra of LumPs (0.9 mg/mL) in standard buffer at 22 °C: *P. phosphoreum* A13 (––); *P. leiognathi* A2D (—). (Bottom) Absorption spectra in 6 M guanidine hydrochloride, pH 7, 22 °C: 6,7-dimethyl-8-ribityllumazine (––); *P. leiognathi* A2D LumP, 0.9 mg/mL (—).

ϵ_{420} = 10 100 M^{-1} cm^{-1}. The ordinate of Figure 1 is scaled appropriately. The concentration of the protein was estimated by the biuret method using trypsinogen as a standard (Goa, 1953). For the native protein the molar concentrations of protein and ligand indicate a binding stoichiometry close to unity.

The visible fluorescence spectra of the three purified LumPs are indistinguishable by eye. In order to make a quantitative comparison among these and the bioluminescence spectral

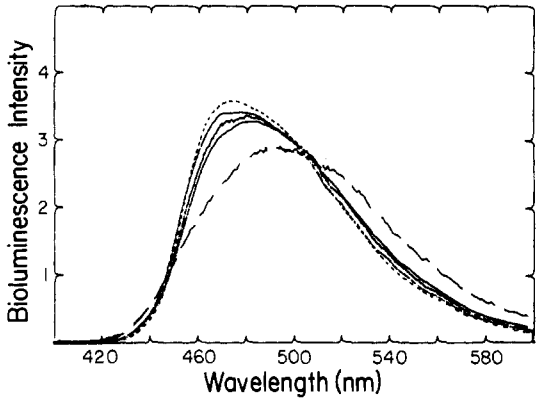

FIGURE 2: In vivo bioluminescence spectra at 22 °C: *P. phosphoreum*, strain A13 (---); *P. leiognathi*, strains A2D, S1, and B477 (—); *P. leiognathi*, strain B475 (– –).

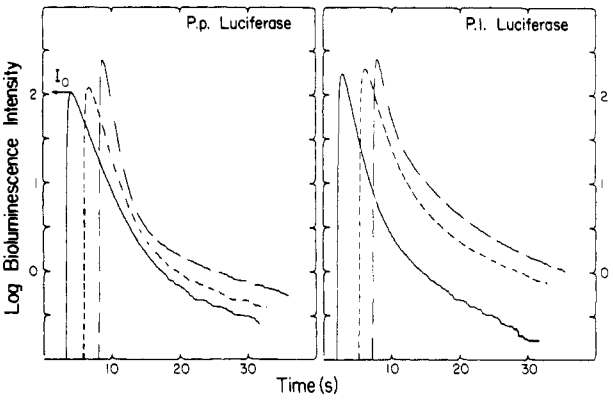

FIGURE 3: Effect of LumP (30 μM) on the bioluminescence kinetics: no LumP (—); *P. leiognathi* (---); *P. phosphoreum* (– –). All reactions were with luciferase (10–15 μM), tetradecanal (50 μM), and $FMNH_2$ (10 μM), in standard buffer with BSA (1 mg/mL), and at 2 °C. (Left) *P. phosphoreum* luciferase, 0.4 μM. (Right) *P. leiognathi* luciferase, 0.7 μM. Bioluminescence intensity is in relative units.

distributions, we have calculated the spectral parameters by moment analysis, and the results are all collected in Table II. For all but the weakest spectra we obtain standard deviations (n = 5–10): maximum, ±1 nm; mean ±0.5 nm; skewness, ±1%; kurtosis, ±1%. The differences among the three purified LumPs' fluorescence (Table II, first three of the top group) are not significant. Even though the S1 type was not purified to minimum absorbance ratio, its fluorescence is as homogeneous as A13. The strain B477 LumP preparation was the one carried over from a previous study (Lee, 1982), but there was an insufficient quantity of it for further workup. Some flavin-like fluorescence contamination is responsible for its spectral broadening and the difference of its parameters, except for its spectral maximum, from those of the three purified LumPs.

In Vivo Bioluminescence Spectra. Figure 2 shows that the bioluminescence spectra of the types of *Photobacterium* from which LumPs have now been purified are distinguishable, with maxima ranging from 475 to 483 nm. The bioluminescence from one of the "dim" *P. leiognathi* is also included in this figure. It has a maximum at 492 nm and a spectral distribution typical of the *Vibrio* genus types. In Figure 2 all the spectra have an isoemissive point around 500 nm.

The spectral parameters are in Table II (middle group). The only bioluminescence that is a "close match" to the LumP fluorescence is that of the A13 strain, the first one studied by Gast & Lee (1978). The others diverge and diverge more as the bioluminescence is found at longer wavelengths. All the parameters shift in a monotonic fashion so there is no clear distinction between the bluest A13, the brightest A2D, or the dim *P. leiognathi* and *V. fischeri*. Nevertheless, for easier comparison, the spectra have been combined and grouped together. Among the *P. leiognathi* it is clear that, concomitant with the shift of bioluminescence spectral maximum to the red, there is a decrease in skewness, and an increase in kurtosis, and a decrease in bioluminescence intensity in culture. Systematic studies of strain S1 showed no change in spectral parameters at the lowest cell density giving a measurable spectrum or over the temperature range 18–33 °C (results not shown). A similar constancy has been reported for A13 (Gast & Lee, 1978).

In Vitro Bioluminescence. Figure 3 shows some novel features of the effects of LumP on the in vitro bioluminescence kinetics. The decay of bioluminescence is complex, consisting initially of a fast first-order process, rate k (Table III), followed by a slower process (or processes) (Lee et al., 1974; Lee & Murphy, 1975; Matheson & Lee, 1983). The left panel of Figure 3 is the reaction with *P. phosphoreum* luciferase. *P. phosphoreum* LumP increases I_0 and the initial decay rate as previously observed (Gast & Lee, 1978). The new observations are that the contribution of the slower process to the total light emission is also enhanced and much more so for the reaction with *P. leiognathi* luciferase (right panel). In both reactions the slow decay rate is unchanged by LumP. Also *P. phosphoreum* LumP is more effective with both luciferase reactions than is the *P. leiognathi* LumP.

Table III: Bioluminescence Properties of Lumazine Proteins[a]

source of luciferase	source of LumP	concn (μM)	initial intensity[b] (10^{12} photons·s^{-1})	decay rate[c] k (s^{-1})	spectral max (nm)
P. phosphoreum	*P. phosphoreum*	0	18	0.068	495
		10	38	0.13	479
		30	52	0.19	477
P. phosphoreum	*P. leiognathi*	10	19	0.079	484
		30	24	0.11	478
P. leiognathi	*P. phosphoreum*	0	38	0.15	495
		10	52	0.16	479.5
		30	53	0.12	475
P. leiognathi	*P. leiognathi*	10	39	0.14	483
		30	41	0.11	481
		75	20	0.05	480

[a] In vitro reaction at 2 °C with $FMNH_2$ and tetradecanal; reaction protocol as described under Materials and Methods. [b] I_0; see Figure 3. [c] Fit to points 0.9 I_0–0.1 I_0.

Numerical results are listed in Table III. The largest kinetic effect is for *P. phosphoreum* luciferase together with its LumP—about a 3 times increase in I_0 and k (Gast & Lee, 1978). Also *P. phosphoreum* LumP is more effective for making the spectral shift than is *P. leiognathi* LumP, and interestingly, for *P. leiognathi* luciferase, even a 75 μM concentration fails to shift the spectral maximum all the way to correspond to the in vivo bioluminescence maximum at 475 nm. The reason for the *decrease* in k here is because of the increasing contribution of the slow process (the kinetics are not analyzed in detail). By 75 μM the bioluminescence is mostly by the slow process. However, this concentration is about 3 times higher than what we estimate to be physiological.

The bioluminescence quantum yield is increased by about 60% for all combinations of luciferases and LumPs (30 μM) under these reaction conditions. At room temperature the increase is about 3-fold as Gast & Lee (1978) first observed

for the A13 luciferase–LumP combination.

Parameters for the in vitro spectra are listed in Table II. Only the reaction of *P. leiognathi* luciferase with its homologous LumP is reported here. Also, with different concentrations of LumP these in vitro spectra (not shown) have an isoemissive wavelength at 500 nm, close to that of the in vivo *Photobacterium* spectra (Figure 3; Lee, 1982).

Reactions with *Vibrio* luciferases and *P. leiognathi* LumP are not shown since the result was similar to the effect of *P. phosphoreum* LumP (Lee, 1982). The *V. harveyi* bioluminescence spectrum is less shifted by *P. leiognathi* than by *P. phosphoreum* LumP, and *V. fischeri* is not affected at all.

Discussion

P. leiognathi contains a lumazine protein with similar properties and in amounts comparable to the one previously identified in *P. phosphoreum*. Improved purification protocols allow recoveries of LumP of up to 60% of that estimated to be present in extracts, a considerable improvement over earlier methods (12%; Small et al., 1980). The product also has slightly better spectral homogeneity. An important innovation (see supplementary material) is the use of a zwitterionic detergent, CHAPS, in the elution buffer on gel exclusion, which improves the separation of LumP from luciferase. LumPs may now be readily purified in 100-mg amounts, thus facilitating the study of their primary and three-dimensional structure as well as other (e.g., NMR) properties.

The chemical structure of the ligand bound to LumPs from *P. leiognathi* or *P. phosphoreum* is the same. This was shown by the identity of Raman fingerprints (Vervoort et al., 1983) and is supported herein by the absorption and fluorescence data (Figure 2 and Table II). The two types of protein have similar Stokes radii since they elute at the same volume on a gel exclusion column. The two proteins differ in that the *P. leiognathi* one is more acidic since it elutes at a higher salt concentration on ion-exchange chromatography, it also has a lower p*I* (O'Kane & Lee, 1985), and it exhibits a tighter binding of the ligand and greater stability at higher temperature (Lee et al., 1985), and the two proteins differ quantitatively in their in vitro bioluminescence properties. Furthermore, the visible absorption bands of the two proteins are slightly shifted from each other although their fluorescence spectra are practically identical. This would be accounted for if the ligand, when excited to its first electronic state, found itself in different environments between the two proteins but had identical interactions in the ground state. This explanation is consistent with the identity of the ring mode frequencies of the lumazine bound to the two proteins (Vervoort et al., 1983).

The bright clones of *P. leiognathi* yield LumP in amounts comparable to *P. phosphoreum*, and therefore they may be classified similarly as 6,7-dimethyl-8-ribityllumazine "overproducers". Evidently this overproduction is for the same function in the two organisms, that is, emission of bioluminescence, as demonstrated by the similarlity in spectral and bioluminescence properties of the two proteins. Although only a few strains have been studied here, it is reasonable to suggest that, from the similarity of their bioluminescence spectra (Table II), LumP is produced and has an emitter function in all brightly bioluminescent *Photobacteria*. A simple test for LumP that can be applied to crude extracts is not available. LumPs are only weakly antigenic (O'Kane, unpublished data), so the very useful microcomplement fixation method (Baumann et al., 1983) is not feasible. However, the blue shifting of the in vitro bioluminescence spectrum by reaction of concentrated cell extract (Small et al., 1980) could be a useful guide.

Bacteria that have different bioluminescence spectra produce LumPs having the same fluorescence spectra. The simple explanation for the different bioluminescence spectra in Figure 2 is that, whereas LumP is the main emitter, there is another contribution to the bioluminescence spectral envelope, something with a spectral maximum further to the red. In the *P. leiognathi* strains going from A2D to B477, as judged from the yields of LumP in extracts, the intracellular concentration of LumP must decrease by at least a factor of 10, and this second emitter therefore contributes a larger share to the spectral envelope, and the bioluminescence maximum shifts to longer wavelength (Table II). Both these emitters must be pumped by a donor species of energy in excess of the 0–0′ transition (~440 nm) of LumP (Ziegler & Baldwin, 1981; Lee et al., 1981). The suggestion that energy flows from the longer wavelength emitter to LumP (Hastings et al., 1981; Hastings & Tu, 1981; Kurfurst et al., 1984) is energetically not feasible (Muller, 1981; Lee, 1982). The overlap integral between the LumP absorption and the in vitro bioluminescence emission from luciferase alone is too small, and moreover, the LumP increases the bioluminescence yield from the reaction.

The in vitro bioluminescence results are a good model for explaining the type-dependence in vivo bioluminescence spectra. At the 10 and 30 μM concentrations of LumP the in vitro bioluminescence spectra with *P. leiognathi* luciferase are identical with the in vivo bioluminescence spectra of B477 and S1, respectively (Table II). Also the in vitro spectra with different concentrations of LumP have an isoemissive wavelength near to that of the in vivo spectra. These spectral data also are consistent with, but are not proof for, the long-wavelength acceptor being the luciferase-generated fluorescent species (Matheson & Lee, 1983), because there are other fluorophores present in the cell extract that qualify on the basis of their fluorescence spectra. The luciferase reaction, being a sensitized type of chemiluminescence, is capable of utilizing a variety of acceptors (Matheson & Lee, 1981; Matheson et al., 1981).

The in vitro bioluminescence properties of the LumPs depend both on the type of luciferase and on the species of LumP (Figure 3; Lee, 1982). The complex decay of bioluminescence intensity is observed for both *Photobacterium* and *Vibrio* luciferases (Lee & Murphy, 1975; Lee et al., 1974; Matheson & Lee, 1983) and has been explained in the case of *V. harveyi* bioluminescence by a kinetic model invoking fast and slow bioluminescence processes. Without a more systematic study we will not try to fit the LumP effect to this model at this time except to use it to provide a qualitative explanation. The model proposes that the bioluminescence processes are kinetically distinct and may utilize different acceptors. Gast & Lee (1978) showed that LumP stimulated I_0 and increased k for *P. phosphoreum* luciferase, as confirmed here. The new observations are that the slower bioluminescence process is increased in intensity but not in rate of decay and that this increase is much more substantial (×10) for *P. leiognathi* luciferase. According to the model the slow decay is controlled by the rate of a dark process, and the bioluminescence pathway is minor. It can be proposed that LumP simply provides a higher concentration of fluorescent acceptor, without competing kinetically with this dark process.

There is also a clear quantitative difference between the effectiveness of the two LumPs (Table III). The difference could simply be electrostatic in origin since both luciferase and LumP are anionic proteins, and to achieve its bioluminescence effect some sort of collision between the two is surely involved (Ward, 1979; Visser & Lee, 1982). Since the *P. leiognathi*

LumP is more negatively charged at pH 7 than the *P. phosphoreum* one, a higher concentration of the former is required to have the same effect as the latter. Equilibrium complexation is very weak in the *Photobacterium* systems (Visser & Lee, 1982) and is not kinetically significant (Lee, 1982). Stronger and kinetically significant complexation is observed with *V. harveyi* luciferase and *P. phosphoreum* LumP (Visser & Lee, 1982; Lee, 1982). The importance of electrostatic forces can be inferred from the fact that phosphate and other anions strengthen the complexation and increase the bioluminescence effectiveness in all these systems (Visser & Lee, 1982; Lee, 1982; Visser et al., 1985).

The *Vibrio* genus types also exhibit spectral variability, and the same explanation of mixed emitters can be proposed for their bioluminescence. Matheson et al. (1981) suggested that a sensitized chemiluminescence mechanism should be applicable to all the types of luminous bacteria. Indeed *V. harveyi* luciferase "recognizes" LumP (Visser & Lee, 1982; Lee, 1982), and at least for one species, the yellow strain of *V. fischeri* (Ruby & Nealson, 1978), a protein having a "sensitizer" function, analogous to LumP, has been reported (Leisman & Nealson, 1982). Although some *Vibrio* bioluminescence spectra differ in vivo to in vitro, those of *V. harveyi* strains do not. Matheson & Lee (1983) suggest that the emitter here is the fluorescent transient species formed with luciferase. The absence of a specialized sensitizer protein would appear to be consistent with the low output of bioluminescence intensity in *V. harveyi*.

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SUPPLEMENTARY MATERIAL AVAILABLE

Detailed protocols for the purification of the three LumPs, including two tables and three figures (19 pages). Ordering information is given on any current masthead page.

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Chemical Characterization of Lumazine Protein from *Photobacterium leiognathi*: Comparison with Lumazine Protein from *Photobacterium phosphoreum*†

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ABSTRACT: The properties of lumazine proteins purified from the marine bioluminescent bacteria *Photobacterium phosphoreum*, a psychrophile, and *Photobacterium leiognathi*, a relatively thermophilic species, are compared. An accurate 1:1 stoichiometry of binding of the ligand 6,7-dimethyl-8-ribityllumazine to each lumazine protein is established by back-titration of the apoprotein with the authentic ligand, using both fluorescence and absorption measurements. Neither protein contains metal cofactors, organic phosphorus, or carbohydrate. Both proteins are anionic and hydrophilic. They each contain a single Trp residue and have blocked amino terminals but otherwise differ in amino acid composition and other properties (*P. phosphoreum* and *P. leiognathi*, respectively): Met (internal), 1, 2; Cys, 2, 1; Arg, 4, 7; p*I*, 4.78 and 4.83, 4.38 and 4.45; M_r, 19750, 21300. In the *P. phosphoreum* protein both Cys residues are accessible, but in the *P. leiognathi* protein the single Cys is "buried". Modification of this buried Cys and at least one Cys in the *P. phosphoreum* protein prevents binding of the ligand. The UV and visible absorption spectra of both lumazine proteins denatured in 6 M guanidine hydrochloride can be accurately modeled by using the number of equivalents of the lumazine derivative and blocked aromatic amino acid model compounds determined by chemical and spectrophotometric analyses for Trp, Tyr, and Phe.

The purification and biological properties of lumazine proteins (LumPs)[1] from two species of the marine bioluminescent bacteria of the genus *Photobacterium* were described in the preceding paper (O'Kane et al., 1985). These proteins enhance the photon output of bacterial luciferases in vitro (Gast & Lee, 1978; O'Kane et al., 1985) and give rise to spectral parameters of the in vitro reaction that match the in vivo spectra of several strains of *Photobacterium* (O'Kane et al., 1985). The characterization of LumP isolated from *Photobacterium phosphoreum*, a psychrophile, has been reported (Small et al., 1980). This species is found to occur at ambient ocean temperatures of 5 °C (FitzGerald, 1978; FitzGerald & Lee, 1978). *Photobacterium leiognathi* strains have also been shown to have LumP (Lee, 1982; Vervoort et al., 1983; O'Kane et al., 1985). These bacteria are normally grown at 28–30 °C in the laboratory and are found to occur naturally at warm ocean temperatures (Yetinson & Shilo, 1979; Shilo & Yetinson, 1979). Both types of LumP, one from a psychrophile and one from a relatively thermophilic species, have similar spectral and biological properties. In this paper we report the chemical characterization of *P. leiognathi* and *P. phosphoreum* LumPs to point out both the similarities and differences in proteins that perform the same function in bacteria that have evolved in very different environments.

Materials and Methods

Authentic 6,7-dimethyl-8-ribityllumazine (Lum) was generously provided by Prof. H. C. S. Wood (University of Strathclyde). "Sequenal" grade guanidine hydrochloride (Gdn·HCl), [bis(trifluoroacetoxy)iodo]benzene (BTI), and micropolyamide plates (5 × 5 cm) were obtained from Pierce Chemical Co., Rockford, IL. 5-(Dimethylamino)-naphthalene-1-sulfonyl chloride (DNS-Cl), 10% DNS-Cl on Celite, and cycloheptaamylose were obtained from Calbiochem-Behring, San Diego, CA. Tryptophan (Gold Label, >99+%) was purchased from Aldrich Chemical Co., and quinine sulfate dihydrate (standard reference material) was obtained from the National Bureau of Standards, Washington, DC. Trypsinogen (1× crystallized), bovine serum albumin (3× crystallized), *N*-acetyltryptophanamide (NAWA),[2] *N*-acetyltyrosinamide (NAYA), glycylphenylalaninamide acetate salt (GPA), 5,5′-dithiobis(2-nitrobenzoic acid), and DEAE-Sepharose were purchased from Sigma Chemical Co., St. Louis, MO. Chicken egg white lysozyme (6× crystallized) was obtained from Miles Laboratories, Kankakee, IL, while glucagon was the product of the Chemical Dynamics Corp.,

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[1] Abbreviations: LumP(s), lumazine protein(s); Lum, 6,7-dimethyl-8-ribityllumazine; Gdn·HCl, guanidine hydrochloride; BTI, [bis(trifluoroacetoxy)iodo]benzene; DNS-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; BSA, bovine serum albumin; NAWA, *N*-acetyltryptophanamide; NAYA, *N*-acetyltyrosinamide; GPA, glycylphenylalaninamide acetate salt; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HSA, human serum albumin; RNase, ribonuclease; EDTA, ethylenediaminetetraacetate; 2-ME, 2-mercaptoethanol; TNB^-, 5-thio-2-nitrobenzoate; $\bar{\lambda}$, mean wavelength position; Tris, tris(hydroxymethyl)-aminomethane.

[2] Although *N*-acetyltryptophanamide is frequently abbreviated as NATA, single letter abbreviations are used for tryptophan, W, and tyrosine, Y, in the blocked aromatic model compounds to avoid confusion.

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