Interaction of *Photobacterium leiognathi* and *Vibrio fischeri* Y1 Luciferases with Fluorescent (Antenna) Proteins: Bioluminescence Effects of the Aliphatic Additive[†]

Valentin N. Petushkov,[‡] Martijn Ketelaars,[§] Bruce G. Gibson,[§] and John Lee*[,§]

Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602, and Institute of Biophysics, Academy of Sciences of Russia (Siberian Branch), 660036 Krasnoyarsk, Russia

Received April 15, 1996; Revised Manuscript Received June 4, 1996[⊗]

ABSTRACT: The kinetics of the bacterial bioluminescence reaction is altered in the presence of the fluorescent (antenna) proteins, lumazine protein (LumP) from *Photobacterium* or the yellow fluorescence proteins (YFP) having FMN or Rf bound, from *Vibrio fischeri* strain Y1. Depending on reaction conditions, the bioluminescence intensity and its decay rate may be either enhanced or strongly quenched in the presence of the fluorescent proteins. These effects can be simply explained on the basis of the same protein−protein complex model that accounts for the bioluminescence spectral shifts induced by these fluorescent proteins. In such a complex, where the fluorophore evidently is in proximity to the luciferase active site, it is expected that the on−off rate of certain aliphatic components of the reaction should be altered with a consequent shift in the equilibria among the luciferase intermediates, as recently elaborated in a kinetic scheme. These aliphatic components are the bioluminescence reaction substrate, tetradecanal or other long-chain aldehyde, its carboxylic acid product, or dodecanol used as a stabilizer of the luciferase peroxyflavin. No evidence can be found for the protein−protein interaction in the absence of the aliphatic component.

When molecular oxygen and $FMNH_2$ react on bacterial luciferase (E; 77 kDa), a metastable product is formed (Scheme 1, intermediate II), called luciferase peroxyflavin (4a-peroxy-4a,5-dihydro-FMN; FMNH-4a-OOH) [for reviews, see Lee et al. (1991a), Baldwin and Ziegler (1992), and Tu and Mager (1995)]. Bioluminescence emission is obtained on the addition of a long-chain aldehyde (RCHO) to luciferase peroxyflavin. It is postulated that a luciferase-bound flavin, 4a-peroxyhemiacetal (III), is the next chemical intermediate (Eberhard & Hastings, 1972; Macheroux et al., 1993) followed by a highly fluorescent species, called the fluorescent transient (FT)[1] (Matheson & Lee, 1983). Since the spectral distribution of the bioluminescence (maximum 495 nm) and the fluorescence of the fluorescent transient are the same, it is concluded that the excited state of this molecule is the origin of the bioluminescence. A luciferase-bound 4a-hydroxy-4a,5-dihydro-FMN (FMNH-4a-OH) has been proposed for its structure (Kurfürst et al., 1984). The final products of the reaction are FMN, H_2O, and RCOOH.

Two other proteins have been discovered that also can influence the bioluminescence process. One is called lumazine protein (LumP; 21 kDa) from *Photobacterium* (Gast & Lee, 1978) and the other, a related yellow fluorescence protein (YFP; 23 kDa) from a yellow bioluminescence strain (Y1) of *Vibrio fischeri* (Macheroux et al., 1987; Daubner et al., 1987). If one of these proteins is included in the reaction, the bioluminescence kinetics is changed and the bioluminescence spectrum no longer corresponds to the fluorescence of the fluorescent transient but is shifted to shorter wavelength (maximum 475 nm), for lumazine protein, or to longer wavelength (maximum 542 nm), for the yellow fluorescence protein.

The physical mechanism of these spectral shifts (time scale, nanoseconds) has been investigated in detail (Lee, 1993; Petushkov et al., 1995a,b, 1996). It has been found that lumazine protein and yellow fluorescence protein can form a long-lived and tight complex with one of the intermediates in the bioluminescence reaction. The existence of this complex in the case with lumazine protein was first found by time-resolved anisotropy experiments (Lee, 1993; Lee et al., 1989a,b). Within this complex the flavin of the fluorescent transient and the lumazine ligand show a weakly coupled dipole−dipole interaction, implying that they have a separation of only about 15 Å. The evidence of this was from a rapid loss of emission anisotropy that occurs on excitation into the flavin absorption band. More direct and precise energy transfer effects were investigated in the experiments with yellow fluorescence protein. The weak dipole−dipole coupling interpretation received strong support by the finding that the energy transfer rate constant is proportional to the donor−acceptor spectral overlap, predicted by theory (Förster, 1948; Petushkov et al., 1996). Both fluorescence dynamics and chromatography experiments have shown that for unreacted luciferase and the corresponding lumazine protein of the same species, *Photobacterium leiognathi*, no complex is produced. Lumazine protein

[†] This work was supported in part by NIH Grants GM-28139 and TW00030 and in part by a grant from Russian state research program 08.05 Newest methods of bioengineering, subprogram engineering in Enzymology Grant N 5-184/o.

* Corresponding author. FAX: (706) 542-1738. Email: jlee@uga.cc.uga.edu.

[‡] Permanent address: Academy of Sciences of Russia (Siberian Branch).

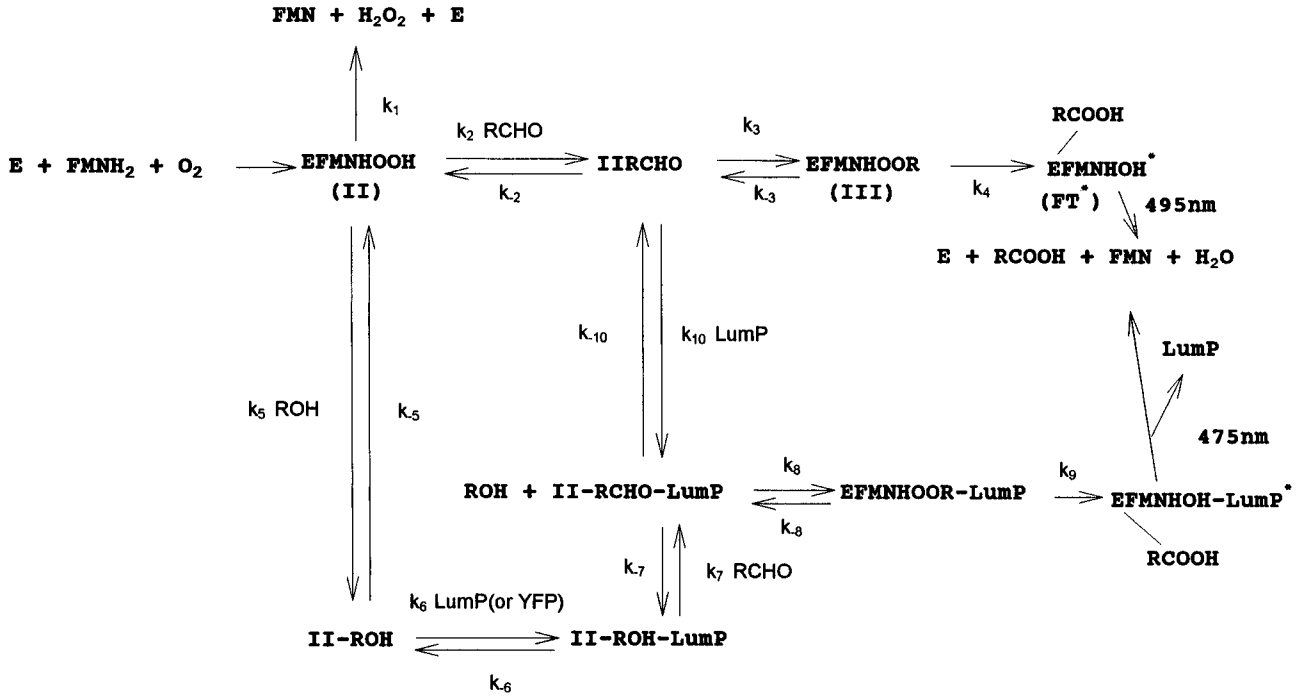
[§] University of Georgia.

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.

[1] Abbreviations: FMN, flavin mononucleotide; LumP, lumazine protein from *Photobacterium leiognathi*; FMN-P, flavin mononucleotide protein from *P. leiognathi*; YFP, yellow fluorescence protein from *Vibrio fischeri* Y1; RFY1-protein and FMNY1-protein, riboflavin and FMN yellow fluorescence protein from *V. fischeri* Y1; antenna protein, LumP or YFP; FT, fluorescent transient.

S0006-2960(96)00893-8 CCC: $12.00

Scheme 1



interacts only with the reaction intermediates of luciferase (Petushkov et al., 1995a). The unusual negative temperature coefficient found recently for the *V. fischeri* Y1 luciferase reaction in the presence of YFP was also suggested to be attributable to protein−protein interaction (Sirokman et al., 1995).

The kinetics of bacterial bioluminescence is not simple, and there is no accurate knowledge of the steps in the reaction sequence. One problem is that one required substrate is a long-chain aliphatic aldehyde, a very hydrophobic substance, and its concentration in the reacting solution is difficult to control because of the equilibrium among homogeneous and micellar states, as well as emulsion, evident by the cloudiness of the solution. Another complication is that bacterial luciferases are very sensitive to aldehyde substrate inhibition (Holzman & Baldwin, 1983; Lei et al., 1994). It is problematic whether multiparameter fitting of the bioluminescence kinetics under such solution conditions can lead to a valid reaction sequence.

The question to be addressed in this work is how the lumazine protein or the yellow fluorescence protein should be incorporated into the bioluminescence kinetic scheme (time scale >0.1 s). The largest kinetic effect was reported for *V. fischeri* Y-1 luciferase and yellow fluorescence protein, a 10 times increase in the initial bioluminescence intensity (I_0) (Eckstein et al., 1990). For *Photobacterium phosphoreum* luciferase with *P. phosphoreum* lumazine protein, I_0 and the rate of decay, *k*, of bioluminescence intensity are increased up to three times (Gast & Lee, 1978; O'Kane et al., 1985; O'Kane & Lee, 1986). For the *P. leiognathi* luciferase reaction, although the *P. leiognathi* lumazine protein is competent in the property of spectral shifting, its effect on bioluminescence kinetics and intensity is not so clear. Conversely, for the luciferase and the so-called "blue fluorescence protein" (BFP) from *V. fischeri* Y-1 (Karatani et al., 1992), kinetic effects are observed but without any modification of the bioluminescence spectrum.

In this present work we will show that both previous kinetic results and new observations can all be explained by the protein−protein complex model previously formulated from the spectral studies. As the lumazine protein or the yellow fluorescence protein must be nearby the luciferase active site, structural interference with the aldehyde interaction is clearly implied and can be used to rationalize the observed changes in the kinetics.

MATERIALS AND METHODS

Proteins. The *Escherichia coli* strain BL21 expression host was cultured and the recombinant riboflavin protein extracted and purified as previously described (Illarionov et al., 1994) with some modifications (Petushkov et al., 1995a). The apoprotein was prepared and "recharged" by removing the riboflavin chromophore with 6 M urea and then extensively dialyzing against buffer containing 6,7-dimethyl-8-ribityllumazine. This recombinant protein has the same properties as the authentic lumazine protein (Illarinov et al., 1994). The yellow fluorescence protein with FMN ligand (FMNY1-protein) or riboflavin (RFY1-protein) was prepared as described before (Petushkov et al., 1995b).

Recombinant *P. leiognathi* luciferase was purchased from the Institute of Biophysics, Krasnoyarsk, Russia. The bioluminescence specific activity of this luciferase was comparable to our own preparations from *P. leiognathi* strains. All proteins had 95% purity according to SDS−PAGE, performed using 15% polyacrylamide gels as described by Laemmli (1970). Luciferase concentrations were assayed by absorbance, assuming $\epsilon(280\ \text{nm}) = 85\,000\ \text{M}^{-1}\ \text{cm}^{-1}$; for lumazine protein $\epsilon(420\ \text{nm}) = 10\,300\ \text{M}^{-1}\ \text{cm}^{-1}$, and it was assumed for FMNY1-protein and RFY1-protein that $\epsilon(464\ \text{nm}) = 12\,500\ \text{M}^{-1}\ \text{cm}^{-1}$, the same as for free FMN. FMN was obtained from Sigma Chemical Co. (St. Louis, MO) and purified on a DEAE column.

Standard Luciferase Assay. For experiments at 23 °C, $FMNH_2$ was prepared in a 1 mL syringe by photoreduction of FMN (80 μM) in 50 mM phosphate buffer, pH 7.0, containing 20 mM EDTA. The bioluminescence reaction was initiated by fast manual injection of 0.5 mL of $FMNH_2$

into a solution of 1.0 mL of standard buffer (50 mM P_i, pH 7.0), containing BSA (1 mg/mL), luciferase, aldehyde, and, if added, lumazine protein. The final concentrations were $FMNH_2$, 25 μM; luciferase, 0.05 μM; and lumazine protein, 10−150 μM. The aldehyde was added as 5 μL of saturated aldehyde in ethanol. This format compromises a single-turnover assay since excess free $FMNH_2$ is quickly removed by autoxidation.

Dithionite Luciferase Assay. The bioluminescence of luciferase at 0 °C was measured by the following procedure. To 5 μL of luciferase, 1.25 μM in a 3 mL luminometer vial on ice, was added 5 μL of FMN, 10 μM, and 5 μL of 80 mM sodium dithionite. Bioluminescence was stimulated then by adding 150 μL of standard buffer containing 5 μL of aldehyde-saturated ethanol solution. Immediately, the vial was transferred to the luminometer thermostated at 0 °C. For steady-state bioluminescence experiments, after the dithionite addition 5 μL of 0.1 M dodecanol in ethanol was added and then, if indicated, 5−40 μL of lumazine protein, 940 μM, or 5 μL of yellow fluorescence protein, 25 μM, followed by the aldehyde solution above.

Luciferase Peroxyflavin. The stability of luciferase peroxyflavin at 0 °C was determined by following the rate of decrease of initial bioluminescence intensity on addition of aldehyde, as a function of time. The inactivation by trypsin of the luciferase peroxyflavin was measured in the same way, by addition of trypsin after the aldehyde initiation of bioluminescence

Aldehydes. A saturated solution of aldehyde in ethanol for decanal = 0.3 M and for tetradecanal = 5.7 mM. The solutions of dodecanal and dodecanol in ethanol were prepared at 0.1 M. For the bioluminescence reactions a dilution of aldehyde in H_2O (aldehyde 1:50) was prepared every 3 h from saturated aldehyde in ethanol. The stated concentrations of aldehydes are nominal since they usually exceed the critical micellar concentration.

Instrumentation. Bioluminescence was measured with a Turner, TD-20e luminometer with the sample thermostated at 0 or 23 °C. The bioluminescence decay rate constant k was obtained by fitting to points $0.8I_0-0.2I_0$, where I_0 = initial intensity. The total light was calculated as I_0/k. Absorption spectra were measured at 0 °C with a Hewlett-Packard 8452 diode array spectrophotometer. Fluorescence and bioluminescence spectra were measured with an SLM (Rochester, NY) Model 8000 spectrofluorometer fitted with Glan-Thompson polarizers in the T-configuration. All emission spectra were measured with a 2 nm slit width, and the sample was contained in a thermostated 10 × 3 mm cuvette, with the excitation along the 10 mm direction and the polarizers set in the magic angle position. All spectra were corrected for optical artifacts (e.g., self-absorption) and for the wavelength sensitivity of the instrument (Velapoldi & Mielenz, 1980). The stopped-flow measurements were performed with a temperature-controlled OLIS RSM1000 apparatus (On-Line Instrument Systems, Bogart, GA). The instrument design did not allow simultaneous bioluminescence and absorbance detection or reaction temperatures less than 10 °C. The reactions were run at 10 °C, rather than room temperature, to slow the rate processes for accurate measurement. One syringe contained photoreduced FMN ($FMNH_2$). The other one contained luciferase with sometimes aldehyde and/or lumazine protein. The final concentrations were $FMNH_2$, 20 μM; luciferase, 50 μM; lumazine protein, 10 μM; and decanal, 500 μM.

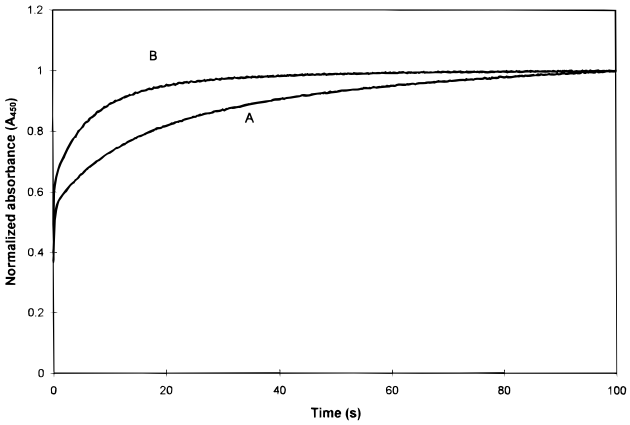


FIGURE 1: Kinetics of formation of free FMN in the bioluminescent reaction of luciferase peroxyflavin and decanal with (B) and without (A) the lumazine protein (10 μM), followed by absorbance at 450 nm (10 °C).

RESULTS

Stopped Flow. In the first stopped-flow experiments (not shown) no aldehyde was added to the reaction mixture. One syringe contained air-saturated buffer with luciferase and the other $FMNH_2$. The reaction kinetics showed the formation of luciferase peroxyflavin with absorption maximum at 380 nm, as was shown in a similar experimental setup before (Faini et al., 1976). Since no aldehyde was present, this intermediate only breaks down to H_2O_2 and free FMN (Scheme 1, k_1 = step 1), as monitored by the increase in FMN absorbance at 450 nm. Employing the global analysis software provided with the OLIS RSM1000, two rate constants were found; $k = 6.3\ s^{-1}$ for the formation of the luciferase peroxyflavin, and $k_1 = 2.6\ s^{-1}$ for its decomposition to FMN. In the absence of luciferase the apparent rate constant for the oxidation of free $FMNH_2$ at 10 °C was about $3\ s^{-1}$.

When lumazine protein was included in the syringe containing the luciferase, there was no effect on the reaction. Both rate constants for the formation and decomposition of luciferase peroxyflavin were the same.

In the next experiments aldehyde was included in the reaction mixture. In order to minimize the inhibitory effect on the bioluminescence, the aldehyde was added to the syringe with luciferase immediately before triggering the reaction. *n*-Decanal was used as the aldehyde because it produces the slowest bioluminescence decay. A slower decay would be predicted to favor the effect of the added lumazine protein.

First the formation of luciferase peroxyflavin could be observed. The next distinguishable species was that of free FMN. Since the absorption spectrum of the fluorescent transient from *P. leiognathi* is not significantly different from luciferase peroxyflavin (Lee et al., 1989a), this species could not be distinguished in this way. The total kinetics at 450 nm (Figure 1, curve A) showed a decrease in the formation rate of FMN compared to $k_1 = 2.6\ s^{-1}$ for the decomposition above without aldehyde.

The inclusion of lumazine protein in the bioluminescence reaction mixture changed the kinetics. The formation of luciferase peroxyflavin monitored by A(380 nm) was not affected but its decomposition rate to FMN, A(450 nm), increased (Figure 1, curve B).

Bioluminescence Kinetics for P. leiognathi. The single-turnover bioluminescence reaction of luciferase is produced

Table 1: Single Turnover Bioluminescence Reaction of *P. leiognathi* Luciferase[a]

additions	decay rate k (s^{-1})	initial intensity I_0	total light I_0/k
5 μL of C14	1.58	100	100
+12 μM LumP	2.15	174	128
5 μL of C14 (1:50)	0.69	100	100
+12 μM LumP	0.85	230	187
5 μL of C14 + 5 μL of C12ol	0.50	100	100
+12 μM LumP	0.66	213	161
5 μL of C10	0.33	100	100
+12 μM LumP	0.36	236	217
20 μL of C10 (1:50)	0.61	100	100
+12 μM LumP	0.57	405	434

[a] Standard luciferase assay (see Materials and Methods). In each reaction without lumazine protein, the initial intensity and total light were set at 100. C10 = decanal; C14 = tetradecanal; C12ol = dodecanol; LumP = lumazine protein and luciferase from *P. leiognathi*, 23 °C, 50 mM P_i, BSA buffer, pH 7.0. Aldehyde (1:50) is a dilution of aldehyde in H_2O 1:50 prepared every 3 h from saturated aldehyde in ethanol. Standard deviations for determining k and I_0 were less than 10%.

Table 2: Influence of Lumazine Protein on Bioluminescence Reactions of *P. leiognathi* Luciferase[a]

additions			decay rate	initial intensity	total light
C10 (mM)	C12ol (mM)	LumP (μM)	k (min^{-1})	I_0	I_0/k
1.7			1.2	100	100
8.3			5.9	300	61
1.7		23	1.5	260	208
1.7	3		0.7	100	100
1.7	3	23	0.45	160	250

[a] Decanal (C10) and dodecanol (C12ol) were prepared from a saturated solution in ethanol. These were added to luciferase peroxyflavin under the conditions of the dithionite luciferase assay. Reaction conditions were otherwise the same as in Table 1, except at 0 °C and the total volume of reagents in the cuvette was about 180 μL. Standard deviations for determining k and I_0 were less than 10%.

by the rapid addition of $FMNH_2$ to a mixture of luciferase and aldehyde. The bioluminescence intensity rises to a maximum I_0, reflecting the overall reaction rate, and then decays at a rate k. In Table 1, for the pairwise comparisons, the initial intensity and total light without lumazine protein have each been set to 100. All values listed here and in Table 2 are averages of 10 measurements with standard deviation around 10%. In each case about 5 μL of saturated aldehyde in ethanol (final concentrations, 20 μM for tetradecanal and 1 mM for decanal) is required to produce the maximum I_0. As is well-known, both I_0 and k depend on the chain length of the aldehyde as well as its concentration but the total light is approximately independent of chain length. The addition of lumazine protein, however, produces a variable effect, increasing both I_0 and k for the tetradecanal case but only I_0 for the decanal reaction. When the aldehyde concentration is limiting (80 μM decanal, 0.4 μM tetradecanal), lumazine protein has no effect on k but causes a strong increase in I_0 for the decanal reaction.

With aldehyde concentration limiting, the rate of decay of bioluminescence intensity (k) is a consequence of a competition between the aldehyde binding (k_2[RCHO]) and the loss of luciferase peroxyflavin through the breakdown to H_2O_2 and free FMN (Scheme 1, step 1). Table 1 shows that the addition of lumazine protein always increases the total light, meaning that the interaction with lumazine protein

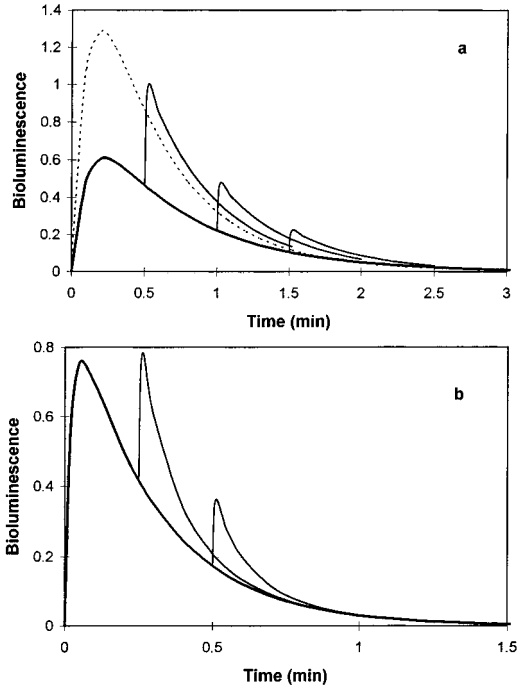


FIGURE 2: (a) Effect of delayed addition of lumazine protein (LumP) on the bioluminescence of luciferase. Three experiments are presented together; LumP (5 μL, final concentration 23 μM) was added secondarily 0.5, 1.0, or 1.5 min after initiation of the reaction with $FMNH_2$, using 0.03 μM luciferase and 8 mM decanal. The dotted line is for LumP present in the reaction at zero time and the bottom solid line without LumP. Conditions: 0 °C, 50 mM P_i, BSA (1 mg/mL) buffer, pH 7.0. (b) Same reactions as for (a) except with 3 mM dodecanal. The ordinate scale is the same, and the reaction with LumP present at zero time (not shown) is the same as the dotted line in (a).

is not rate limiting; in Scheme 1, $k_{-2} + k_3 \ll k_{10}$[LumP] under any of the conditions used.

Table 2 contains the results for the single-turnover conditions induced by the dithionite reaction. If aldehyde is not in the initial reaction mixture, the luciferase peroxyflavin decays at a rate of 1.5 min^{-1} (0 °C). Inclusion of lumazine protein produces an identical decomposition rate giving no evidence of protein−protein interaction at this point, whereas addition of the dodecanol solution (5 μL) as reported by Tu (1979) markedly stabilizes the luciferase peroxyflavin ($k_{-5} < 0.02$ min^{-1}).

Table 2 shows that the dodecanol also allows for interaction with lumazine protein. For the dithionite reaction, about 5 μL of 6 mM decanal (final concentration 170 μM) is required for maximum bioluminescence intensity. For lower amounts (33 μM), the bioluminescence decay rate, k, is in the same range as the decomposition rate of the luciferase peroxyflavin, but lumazine protein still magnifies both the I_0 and the total light, comparable to its effect under the conditions of Table 1.

The increase in total light is easily explained as due to the two times higher fluorescence quantum yield of the lumazine protein over the fluorescent transient. The kinetics implies that the protein−protein interaction is not rate limiting the bioluminescence under these conditions. A clear change occurs, however, if the luciferase peroxyflavin is stabilized with dodecanol. Lumazine protein slows the bioluminescence decay rate, and the enhancement of the I_0 is also reduced.

Figure 2a shows that the delayed addition of lumazine protein has the same effect as when it is present at the

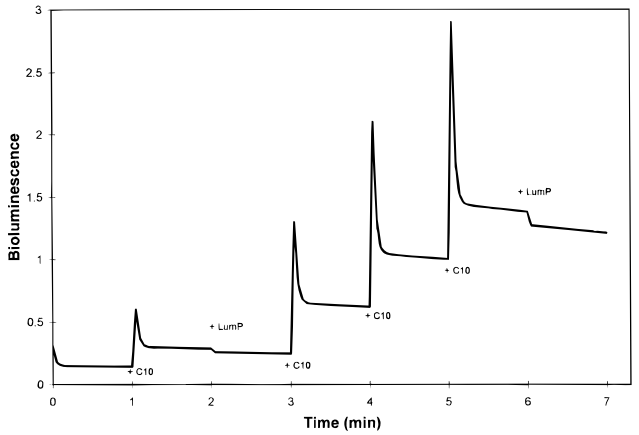


FIGURE 3: Effect of delayed addition of 5 μL of decanal (1:50) at times 1, 3, 4, and 5 min and of LumP at 2 and 6 min on the bioluminescence of luciferase. The reaction was initiated with 5 μL of decanal (1:50). Conditions: 0 °C, 3 mM dodecanol, 50 mM P_i, BSA buffer, pH 7.0. Final concentration of luciferase is 0.03 μM in a total volume about 180 μL.

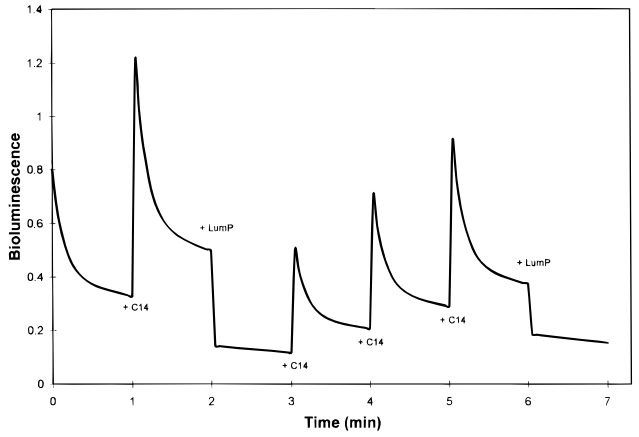


FIGURE 4: Same as Figure 3 except with 110 μM tetradecanal.

beginning of the reaction (dotted line). The solid line starting from the origin is from the dithionite luciferase assay with decanal but without lumazine protein, $k = 1.5$ min^{-1}. Lumazine protein added in separate experiments at 0.5, 1.0, and 1.5 min produces the same bioluminescence decay rate ($k = 2$ min^{-1}) but the I_0 is increased by about 2.5 every time, the same as in Table 2. Figure 2b is for the reaction initiated with dodecanal. Without lumazine protein the decay rate is 3.5 min^{-1}. On addition of lumazine protein the I_0 is again increased about two times with the decay becoming biphasic, 9.0 and 3.5 min^{-1}. With tetradecanal (not shown) the effect of adding lumazine protein is the same, except the decay rate constants are several times faster.

Figures 3 and 4 are for the reaction of the dodecanol-stabilized luciferase peroxyflavin. An approximate steady state is established but the kinetics is quite complex. On addition of decanal or dodecanal there is an initial flash falling to a nearly constant level of bioluminescence intensity. This intensity increases slowly to a maximum after about 10 min and then decays with a half-time of 40 min. This is not apparent on the time scale of Figure 3, and the behavior is also variable, depending, for example, on aldehyde concentration. The initial flash response is a consequence of the aldehyde dilution into the buffer. If the aldehyde is prepared first in the buffer and then is added to luciferase with dithionite and dodecanol, only the steady-state level is seen without the rapid flash.

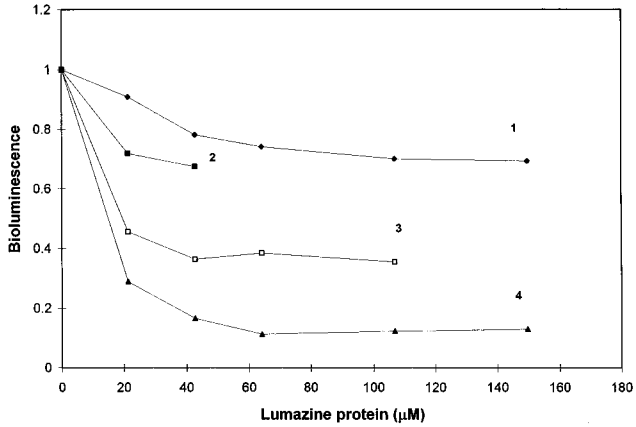


FIGURE 5: Inhibition of the steady-state bioluminescence of *P. leiognathi* luciferase by LumP. Bioluminescence started using 0.03 μM luciferase without LumP, with 3 mM dodecanol, and 5 μL of aldehydes (1, 6 mM decanal; 2, 3, 2 mM dodecanal; 4, 110 μM tetradecanal). Conditions: 0 °C, 50 mM P_i, BSA (1 mg/mL) buffer, pH 7.0; total volume about 180 μL.

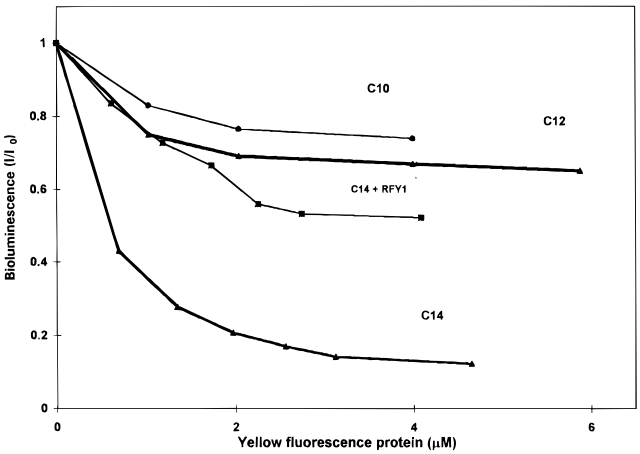


FIGURE 6: Same as Figure 5 except luciferase and FMNY1-protein were from *V. fischeri* Y1. Also shown is the inhibition effect with tetradecanal by RFY1-protein.

The addition of lumazine protein causes a decrease in the steady-state level. For tetradecanal (Figure 4), lumazine protein at 2 min decreases the bioluminescence level 3−4 times, and the decay becomes much slower. The effect is smaller (<10%) for decanal (Figure 3). It is to be noted, however, that after the addition of lumazine protein the next injection of aldehyde causes a severalfold increase in both the flash height and the steady-state level in both cases.

Figure 5 shows the dependence of this inhibition of the bioluminescence on concentration of lumazine protein, for the reaction conditions of Figures 3 and 4. The longer the aldehyde chain length, the greater the inhibition by added lumazine protein (curves 1, 3, and 4). Also the inhibition is less (curve 2) during the time of the initial flash than later (curve 3) into the steady-state reaction phase or the slow decay phase in the case of tetradecanal.

Bioluminescence Kinetics for V. fischeri. Steady-state bioluminescence kinetics experiments similar to those shown in Figures 3−5 were carried out using *V. fischeri* Y1 luciferase and FMNY1-protein or RFY1-protein. The inhibition results are shown in Figure 6 and are comparable to those in Figure 5, i.e., a protein concentration effect dependent on aldehyde chain length and also on the ligand. The RFY1-protein had less effect than FMNY1-protein. The presence BSA at 1 mg/mL concentration did not change the inhibition effect for FMNY1-protein.

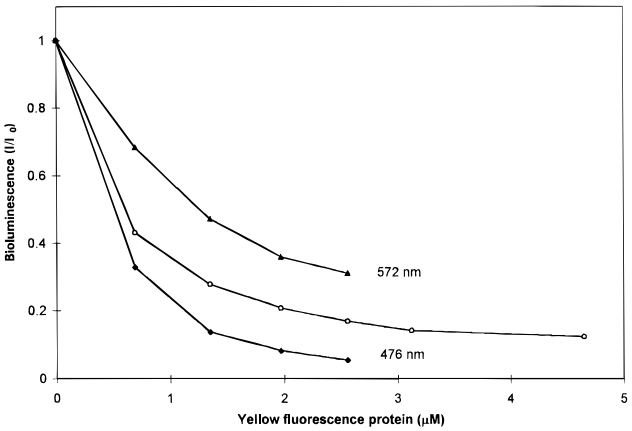


FIGURE 7: Inhibition of the yellow versus blue bioluminescence emissions from the steady-state reaction from *V. fischeri* luciferase, with tetradecanal and dodecanol. Color filters are used to separately detect the 572 nm band (triangles) and the 476 nm band (diamonds). The open circles are without a filter.

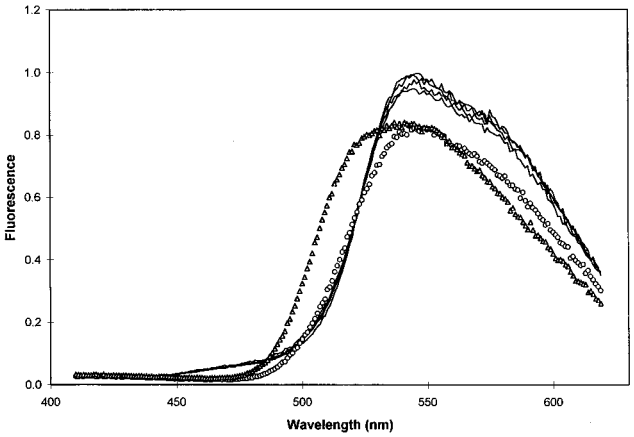


FIGURE 8: Effect of FMNY1-protein on the stability of luciferase hydroxyflavin. The first family (upper set of four curves) is the steady-state fluorescence spectra of a mixture of luciferase hydroxyflavin (from *P. leiognathi*, 10 μM) after addition of FMNY1-protein (15 μM) at 0 °C. These four curves were measured for a fresh (0 h) preparation and then at 1, 2, and 3 h after incubation at 0 °C. The open circles are for the 3 h sample after being warmed to 25 °C, and the triangles are after 40 °C and recooling.

Two bioluminescence emission bands can be distinguished in this reaction. One has a maximum around 495 nm, originating from the fluorescent transient reaction via step 3 in Scheme 1, and the other at 540 nm from the yellow fluorescence protein, via steps 6 through 10 with YFP substituted for LumP. The two bands can be separated with color filters, and Figure 7 shows that the shorter wavelength bioluminescence is much more effectively inhibited than the yellow bioluminescence (measured at 576 nm). Also consistent with the previous results is that FMNY1-protein in the single turnover reaction, and with or without dodecanol, increases the bioluminescence decay rate, seen by comparing the first two rate columns in Table 3.

Luciferase is known to be extremely sensitive to protease inactivation. In Table 3 the inactivation is followed by the influence of added trypsin on the bioluminescence decay rate (k) in the single turnover assay. In line 1, for tetradecanal in the presence of dodecanol, trypsin increases this decay from 0.15 to 2.2 min^{-1}. However, the FMNY1-protein provides some protection against trypsin inactivation, slowing the decay rate to 1.4 min^{-1}. RFY1-protein is not as protective if at all, and FMN-P (*P. leiognathi*) and BSA show no influence. Also without influence were the riboflavin protein (*P. leiognathi*) and the blue fluorescence protein (*V. fischeri*). The remaining lines suggest that the dodecanol is required for the protection property of FMNY1-protein. Also it is apparent that the protection is not aldehyde chain length dependent (lines 1 and 2). The last two lines show that the inactivation rate is approximately linearly dependent on the added trypsin. These results lead to the conclusion that the FMNY1-protein binds the luciferase peroxyflavin stabilized by the dodecanol and in this form the trypsin digestion becomes inhibited.

A final set of experiments was made with other fluorescent proteins. Lumazine protein from *P. leiognathi* at 40 μM concentration shifted the *V. fischeri* Y1 bioluminescence to a shorter wavelength (not shown), the same as for the bioluminescence reaction with *P. leiognathi* luciferase. The riboflavin protein (FFP), FMN protein, both from *P. leiognathi*, and BFP from *V. fischeri*, all at 40 μM, had no effect on steady-state bioluminescence intensity in the *V. fischeri* reaction and had no influence on the bioluminescence spectrum.

FMNY1-Protein Stabilizes Luciferase Hydroxyflavin. Figure 8 is the fluorescence of the luciferase hydroxyflavin (*P. leiognathi*) in the presence of FMNY1-protein. The four solid lines are for the freshly prepared mixture and after 1, 2 and 3 h. There is almost no change after 3 h, in contrast to the fact that the fluorescence transient itself disappears almost completely after 2 h under the same conditions (Petushkov et al., 1995a). Of course, the fluorescence spectrum here is mainly contributed to by the FMNY1-protein itself, so in order to resolve the fluorescent transient contribution, the 3 h sample was heated at 25 °C for 20 min and then recooled (open circles). The intensity at 470 nm

Table 3: Inactivation of Luciferase by Trypsin Shows Protection by Added Fluorescent Protein[a]

					trypsin added				
C14 (μM)	C10 (μM)	C12ol (mM)	na[b]	FMNY1	na	FMNY1	RFY1	FMN-P	BSA
0.3		3	0.15	0.55	2.2	1.4	1.9	2.1	2.1
	160	3	0.1		2.15	1.3			
0.3		0	1.05	1.5	12	10			
15		0	0.3[c]		5.5	4			
	160	0	1.25	1.5	9	10−12			
0.3		0	1.05	1.5	3.6[d]	2.5[d]			3
	160	0	1.1	1.6	3.5[d]	3.8[d]			4

[a] Rates, k (min^{-1}), of bioluminescence decay at 0 °C of peroxyflavin intermediate *V. fischeri* with different aldehydes in the presence of different proteins. [b] na = no addition of fluorescent protein. [c] Slower decay constant; 15 μg of trypsin added. [d] 3 μg of trypsin added. Dithionite assay at 0 °C: 5 μL of luciferase and 5 μL of FMN were mixed with 5 μL of dithionite (80 mM) and then 150 μL of aldehyde in air-saturated buffer. Trypsin was then added (5 μL or [d]1 μL, 3 mg/mL). Final concentrations were luciferase *V. fischeri*, 0.01 μM; FMN, 0.06 μM; FMNY1-protein, RFY1-protein, FMN-P (from *P. leiognathi*), and BSA, all 0.7 μM. Total volume of reagents in the cuvette was about 180 μL.

is now zero and at 545 nm drops by 17%. This difference between the spectra initially and after heating the sample is the contribution arising from energy transfer from the fluorescent transient donor to the FMNY1-protein acceptor, within the protein−protein complex. The long wavelength part still has the same shape, indicating that the FMNY1-protein is still intact. The 470 nm contribution was from the uncomplexed fluorescent transient and is now gone. After heating at 40 °C and recooling, the spectrum (triangles) is almost the same as free FMN.

DISCUSSION

It is clear from the results of our previous work that lumazine protein and the yellow fluorescence proteins are able to form a specific 1:1 complex with the luciferase reaction intermediates (Lee et al., 1991b; Lee, 1993; Petushkov et al., 1995a,b, 1996). It is a simple extension of this knowledge then to say that certain rate processes in the reaction pathway could be affected by the complexation. The major conclusion here is that the aliphatic participant is the "glue" holding the two proteins in place, or it induces a conformational change favoring the interaction. This glue is either the dodecanol stabilizing the luciferase peroxyflavin, the aldehyde substrate, or its carboxylic acid reaction product.

No evidence can be found for interaction in the absence of the aliphatic additive. The rapid scan stopped flow shows that lumazine protein has no influence on either the rate of formation of luciferase peroxyflavin or its decay in the absence of aldehyde. On the other hand, the release of FMN from the luciferase peroxyflavin is faster in its complex with the lumazine protein. However, the possibility of protein−protein interaction without the aliphatic cannot be completely excluded because the luciferase peroxyflavin is not sufficiently stable in the absence of dodecanol to be able to do a chromatography study, as was able to be demonstrated with the stabilized intermediate.

The single-turnover results also indicate that the lumazine protein complex formation is faster than the k_3 bioluminescence process. At whatever time into the reaction the fluorescent protein is added, the reaction pathway is completely directed along the lower part of Scheme 1. A quantitative account, of course, is not feasible without accurate knowledge of the rate constants along the reaction pathway. This information is available for the *V. harveyi* luciferase case (Abu-Soud et al., 1992, 1993), and assuming similar values for two present luciferases under study here, i.e., of order 1 s^{-1} for the first-order rate constants, it is reasonable to propose that the lumazine protein or YFP association rates could easily out-compete these steps.

The second major observation is that the protein−protein complexation markedly slows the exchange between dodecanol and aldehyde. The model proposed to explain the bioluminescence spectral shifts requires that the ligand of the fluorescent protein lies proximate to the luciferase active site. It is reasonable to assume that both dodecanol and aldehyde occupy the same binding site close to the luciferase active site and therefore the exchange between the two must be affected by the physical presence of the second protein. Also, due to its size a longer chain length aldehyde might be more affected than a shorter one. The RFY1-protein is less effective than FMNY1-protein due to its weaker association. The emission in the yellow band is much less attenuated than from the fluorescent transient contribution around 495 nm because the addition of YFP would favor the lower pathway in Scheme 1 at the expense of the upper "blue" pathway.

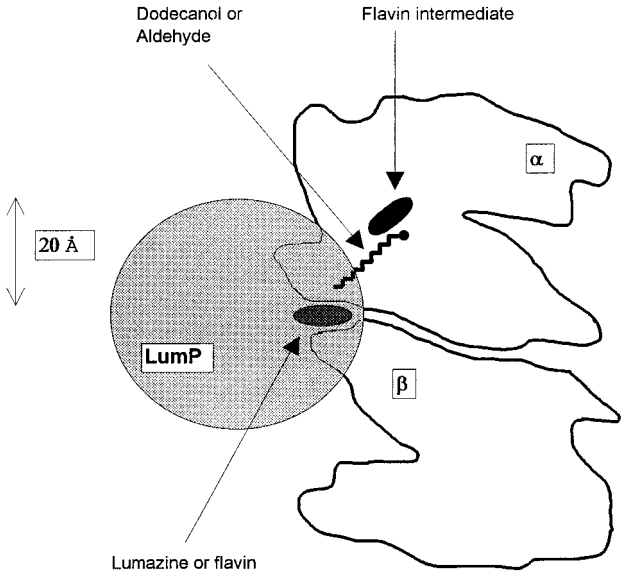


FIGURE 9: In the specific complex formed between the luciferase peroxyflavin and fluorescent (antenna) protein (LumP or YFP), the ligands are positioned so that rapid energy transfer to them from the luciferase flavin donor out-competes the donor fluorescence rate (0.1 ns^{-1}). The antenna protein shown here as LumP partly covers the luciferase aldehyde site. The luciferase shape is traced from the three-dimensional structure [Figure 2 of Fisher et al. (1995)].

From Figures 5, 6, and 7, we suggest that, with dodecanol and tetradecanal (the maximum inhibition effect), lumazine protein and yellow fluorescence protein show an apparent noncompetitive inhibition constant of 7 (LumP) and 0.5 μM (YFP). However, the real interaction for LumP must be with about half this value, because the bioluminescence reaction with lumazine protein goes by steps 7−9 with about twice the quantum yield than by steps 3 and 4. A value of 3.5 μM (for lumazine protein) and 0.5 μM (for yellow fluorescence protein) for the interaction constant is consistent with the concentrations needed for the bioluminescence spectral shifting effects.

Extrapolating now to the reaction pathway, the published model suggests reversible steps including the interaction of the luciferase peroxyflavin with aldehyde (Abu-Soud et al., 1992, 1993; Francisco et al., 1993). If the off-rate of the aldehyde in the protein−protein complex is slowed more than the on-rate, this would lead to a shift of the equilibrium (k_7 and k_8) and a consequent increase in steady-state concentration of the final intermediate (e.g., EFMNHOORLumP) prior to the light step. This would account for the increase in light intensity and rate of its decay as observed, markedly for the FMNY1-protein case (Eckstein et al., 1990). The explanation for the enhancement of the total light in the reaction with lumazine protein is trivial. The fluorescence quantum yield for lumazine protein is almost twice that of the fluorescent transient. For the yellow fluorescence proteins, the fluorescence quantum yield is not as high and the total light therefore remains the same.

We have attempted to summarize our present knowledge about the protein−protein complex in terms of a physical picture in Figure 9. The three-dimensional structure of luciferase has been determined to a resolution of 2.4 Å, but this is not sufficient to locate the flavin in the active site

(Fisher et al., 1995). However, a structurally homologous protein, the nonfluorescent protein, has been solved at a resolution of 1.6 Å but has two bound flavins per subunit of the homodimer, one near the N- and C-terminal partially solvent exposed and the other buried in the subunit interface (Moore & James, 1994, 1995; Li & Meighen, 1994, 1995). Many studies have concluded that the $FMNH_2$ binding site is situated on the α-subunit of luciferase, and this was supported again recently in experiments using two-quantum affinity modification (Benimetskaya et al., 1994). Energy transfer of the excited $FMNH_2$ produced cleavage only on the α-subunit. Arguments have been presented to place the luciferase active flavin at the terminal position as distinct from the interface region.

Figure 9 shows the fluorescent protein partly covering or overlapping the active site for aliphatic substrates (aldehyde, acid, or alcohol). The protease-sensitive site is not known but may also be close to the active site. The aldehyde is strongly bound in the active site along the entire length of the alkyl chain with the strongest interactions at the CHO group (Viswanathan et al., 1979). The aldehyde function being chemically involved with the flavin intermediate must lie at the active site. Thus the chain length which is the variable must be the part that overlaps with the fluorescent protein. Tetradecanal would present more hydrophobic interactions than a smaller size aldehyde. For decanal then, the fluorescent protein is more effective in fixing the aldehyde onto luciferase and makes it less able to dissociate away and, therefore, increases its reaction probability, resulting in an increase in the rate constant for bioluminescence decay. This idea explains the effect of YFP, which increases the decanal-induced bioluminescence decay rate of Y1 *V. fischeri* luciferase 10 times (Eckstein et al., 1990).

The present spectral results imply that, in the protein−protein complex, the peroxyflavin and ligand of the acceptor are separated by about 15 Å. The ligand structure of the fluorescent protein (lumazine or flavin) is also important and specifically required for this interaction in each case, because the apolumazine protein recharged by FMN or riboflavin is ineffective (Petushkov et al., 1995a). At the same time, lumazine protein from *P. leiognathi* is active with luciferase peroxyflavin from *V. fischeri* Y1. Perhaps there is, again by analogy to the nonfluorescent protein, a second binding site on the luciferase for FMN (Li & Meighen, 1995) that provides this specificity for the ligand of the fluorescent protein. It may not be coincidental that this 15 Å distance is about the same as the length of the aldehyde molecule, or other aliphatic additive, and of the separation of the putative flavin sites.

ACKNOWLEDGMENT

We thank professor Dr. Adelbert Bacher, Technical University, Munich, for the generous gift of the lumazine derivative and Professor Dr. Sandro Ghisla, University of Konstanz, for his critical reading.

REFERENCES

Abu-Soud, H., Mullins, L. S., Baldwin, T. O., & Raushel, F. M. (1992) *Biochemistry 31*, 3807−3813.

Abu-Soud, H., Clark, C., Francisco, W. A., Baldwin, T. O., & Raushel, F. M. (1993) *J. Biol. Chem. 268*, 7699−7706.

Baldwin, T. O., & Ziegler, M. M. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. III, pp 467−532, CRC Press, Boca Raton, FL.

Benimetskaya, L. Z., Kozionov, A. L., Novozhilov, S. Yu, Petushkov, V. N., Rodionova, N. S., & Shtokman, M. I. (1994) *Dokl. Akad. Nauk 336*, 114−117 (Russian).

Eberhard, A., & Hastings, J. W. (1972) *Biochem. Biophys. Res. Commun. 47*, 348−353.

Eckstein, J. W., Cho, K. W., Colepicolo, P., Ghisla, S., Hastings, J. W., & Wilson, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1466−1470.

Faini, G. J., DeSa, R. J., & Lee, J. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 82−86, Elseiver, Amsterdam.

Fisher, A. J., Raushel, F. M., Baldwin, T. O., & Rayment, I. (1995) *Biochemistry 34*, 6581−6586.

Förster, T. (1948) *Ann. Phys. 2*, 55−75.

Francisco, W. A., Abu-Soud, H. M., Clark, A. C., & Baldwin, T. O. (1993) in *Flavins and Flavoproteins* (Yagi, Y., Ed.) pp 829−837, Walter de Gruyter & Co., Berlin.

Daubner, S. C., Astorga, A. M., Leisman, G. B., & Baldwin, T. O. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8912−8916.

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

Holzman, T. F., & Baldwin, T. O. (1983) *Biochemistry 22*, 2838−2846.

Illarionov, B. A., Illarionova, V., Lee, J., Van Dongen, W., & Vervoort, J. (1994), *Biochim. Biophys. Acta 1201*, 251−258.

Karatani, H., Wilson, T., & Hastings, J. W. (1992) *Photochem. Photobiol. 55*, 293−299.

Kurfürst, M., Ghisla, S., & Hastings, J. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2990−2994.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

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

Lee, J. (1993) *Biophys. Chem. 48*, 149−158.

Lee, J., O'Kane, D. J., & Gibson, B. G. (1989a) *Biochemistry 28*, 4263−4271.

Lee, J., O'Kane, D. J., & Gibson, B. G. (1989b) *Biophys. Chem. 33*, 99−111.

Lee, J., Matheson, I. B. C., Müller, F., O'Kane, D. J., Vervoort, J., & Visser, A. J. W. G. (1991a) in *Chemistry and biochemistry of flavins and flavoenzymes* (Müller, F., Ed.) Vol. II, pp 109−151, CRC Press, Boca Raton, FL.

Lee, J., Wang, Y., & Gibson, B. G. (1991b) *Biochemistry 30*, 6825−6835.

Lei, B., Cho, K. W., & Tu, S.-C. (1994) *J. Biol. Chem. 269*, 5612−5618.

Li, Z., & Meighen, E. A. (1994) *J. Biol. Chem. 269*, 6640−6644.

Li, Z., & Meighen, E. A. (1995) *Biochemistry 34*, 15084−15090.

Macheroux, P., Schmidt, K. U., Steinerstauch, P., Ghisla, S., Colepicolo, P., Kurfürst, M., & Hastings, J. W. (1987) *Biochem. Biophys. Res. Commun. 146*, 101−106.

Macheroux, P., Ghisla, S., & Hastings, J. W. (1993) *Biochemistry 32*, 14183−14186.

Matheson, I. B. C., & Lee, J. (1983) *Photochem. Photobiol. 38*, 231−240.

Moore, S. A., & James, M. N. G. (1994) *Protein Sci. 3*, 1914−1926.

Moore, S. A., & James, M. N. G. (1995) *J. Mol. Biol. 249*, 195−214.

O'Kane, D. J., & Lee, J. (1986) *Methods Enzymol. 133*, 149−172.

O'Kane, D. J., Karle, V. A., & Lee, J. (1985) *Biochemistry 24*, 1461−1467.

Petushkov, V. N., Gibson, B. G., & Lee, J. (1996) *Biochemistry 35*, 8413−8418.

Petushkov, V. N., Gibson, B. G., & Lee, J. (1995a) *Biochemistry 34*, 3300−3309.

Petushkov, V. N., Gibson, B. G., & Lee, J. (1995b) *Biochem. Biophys. Res. Commun. 211*, 774−779.

Sirokman, G., Wilson, T., & Hastings, J. W. (1995) *Biochemistry 34*, 13074−13081.

Tu, S.-C. (1979) *Biochemistry 18*, 5940−5945.

Tu, S.-C., & Mager, H. I. X. (1995) *Photochem. Photobiol. 62*, 615−624.

Velapoldi, R. A., & Mielenz, K. D. (1980) *NBS Spec. Publ. (U.S.)* No. 260−264.

Viswanathan, T. S., Campling, M. R., & Cushley, R. J. (1979) *Biochemistry 18*, 2504−2508.

BI9608931