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Dynamic Fluorescence Properties of Bacterial Luciferase Intermediates†

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ABSTRACT: Three fluorescent species produced by the reaction of bacterial luciferase from *Vibrio harveyi* with its substrates have the same dynamic fluorescence properties, namely, a dominant fluorescence decay of lifetime of 10 ns and a rotational correlation time of 100 ns at 2 °C. These three species are the metastable intermediate formed with the two substrates $FMNH_2$ and O_2, both in its low-fluorescence form and in its high-fluorescence form following light irradiation, and the fluorescent transient formed on including the final substrate tetradecanal. For native luciferase, the rotational correlation time is 62 or 74 ns (2 °C) derived from the decay of the anisotropy of the intrinsic fluorescence at 340 nm or the fluorescence of bound 8-anilino-1-naphthalenesulfonic acid (470 nm), respectively. The steady-state anisotropy of the fluorescent intermediates is 0.34, and the fundamental anisotropy from a Perrin plot is 0.385. The high-fluorescence intermediate has a fluorescence maximum at 500 nm, and its emission spectrum is distinct from the bioluminescence spectrum. The fluorescence quantum yield is 0.3 but decreases on dilution with a quadratic dependence on protein concentration. This, and the large value of the rotational correlation time, would be explained by protein complex formation in the fluorescent intermediate states, but no increase in protein molecular weight is observed by gel filtration or ultracentrifugation. The results instead favor a proposal that, in these intermediate states, the luciferase undergoes a conformational change in which its axial ratio increases by 50%.

Bacterial luciferase is an enzyme (M_r 77 000; α,β) that reacts with $FMNH_2$, O_2, and a long-chain aliphatic aldehyde to produce bioluminescence (Lee, 1985). The emission spectrum is broad and unstructured, with a maximum in the range of 487-505 nm depending on the type of bacterium from which the luciferase was isolated. Hastings and Gibson (1963) showed that bioluminescence was also produced if the addition of aldehyde was delayed over the other reactants. They proposed that the luciferase-bound $FMNH_2$, which they called intermediate I, reacted with oxygen to form an oxygenated complex, intermediate II.[1] They suggested that the bioluminescence was by a reaction of the aldehyde with II. At room temperature, the bioluminescence potential of II lasted less

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[1] Abbreviations: BSA, bovine serum albumin; ANS, 8-anilino-1-naphthalenesulfonic acid; II, luciferase intermediate II; FT, luciferase fluorescent transient; Q_f, quantum yield of fluorescence; FWHM, full width at half-maximum; DW, Durbin-Watson parameter; τ, fluorescence decay time; ϕ, rotational correlation time; HPLC, high-performance liquid chromatography.

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than a minute, but it was more stable at lower temperature.

The properties of intermediate II have been the subject of a number of investigations because of its supposed key place in the bioluminescence scheme. The most effective method for its stabilization is by inclusion of dodecanol in the reaction buffer (Tu, 1979, 1986). Using dodecanol for stabilizing II for as long as 24 h (2 °C), it has even been possible to obtain the ^{13}C NMR spectrum of II and to characterize its chemical structure as a 4a-substituted dihydro-FMN bound to the luciferase (Vervoort et al., 1986a). Evidence from the NMR of a model compound led these workers to conclude that this substitution was by –O–O– in an almost planar configuration around the C(4a) atom. Hastings et al. (1973) proposed that the substituted group was a hydroperoxide.

The absorption maximum of II is around 370 nm, and it is weakly fluorescent. Balny and Hastings (1975) observed that the fluorescence maximum was around 505 nm (uncorrected) but that on continuous irradiation in the excitation beam of the spectrofluorometer the fluorescence intensity increased several times accompanied by a shift in maximum to a shorter wavelength. Their final fluorescence spectrum was the same as the bioluminescence. Tu (1979), however, reported a contradictory result that this irradiation shifted the fluorescence to longer wavelengths. Both groups reported that the fluorescence enhancement of II did not lead to any loss of its bioluminescence potential (Tu, 1986).

A third fluorescent "intermediate" in this luciferase reaction has also been reported. In the course of the luciferase-catalyzed oxidation of $FMNH_2$ and aldehyde, a fluorescent transient (FT) species is observed having the same fluorescence spectrum as the bioluminescence spectrum, and having a kinetic relationship to the bioluminescence intensity (Matheson et al., 1981; Matheson & Lee, 1983). The same FT was also observed on reaction of II with aldehyde (Kurfurst et al., 1984). Its absorption spectrum has also been reported (Kurfuerst et al., 1987).

In summary at this time, it appears that the absorption maxima of all three of these intermediate species are very close, in the range 360–372 nm. Also, the fluorescence spectrum of FT and, at least according to Balny and Hastings (1975), the spectrum of the high-fluorescence form of II are the same as that of the bioluminescence emitter. The three species seem to differ only in their method of preparation and in fluorescence quantum yield. It was the original purpose of this investigation to resolve the spectral discrepancy mentioned above between the observations of Tu (1979) and of Balny and Hastings (1975), and to extend the characterization to fluorescence anisotropies and lifetimes. The result is that the high-fluorescence form of II produced photochemically and FT are found to have the same dynamic fluorescence properties with a much longer rotational correlation time, 100 ns, than observed for native luciferase, 65 ns (2 °C). An explanation based on an axial ratio difference is proposed.

Materials and Methods

Luciferase was from the aldehyde-deficient mutant MAVA of *Vibrio harveyi*. It was purified as described elsewhere (O'Kane et al., 1986) to high specific activity, around 1.5×10^{14} photons s^{-1} mg^{-1}, as assayed with decanal. The concentration of luciferase was calculated from its absorbance, $\epsilon(280) = 85\,000$ M^{-1} cm^{-1}. Bovine serum albumin (BSA) was from Sigma Chemical Co. (St. Louis, MO) and was purified to the monomer by size-exclusion HPLC (TSK 3000). FMN was also from Sigma, "synthetic 80% by HPLC". Preparative ion-exchange HPLC (TSK-DEAE 3SW) using the conditions specified for the separation of FMN isomers (Van Schagen & Muller, 1981) showed the presence of only the 5′-phosphate. With the "free"-grade (Sigma), the other isomers as well as riboflavin were evident. ANS (8-anilino-1-naphthalenesulfonic acid) was twice recrystallized. This and yeast alcohol dehydrogenase were gifts of Dr. J. Brewer. All other chemicals used were the best commercial grades.

The intermediate was preparedby rapid addition of $FMNH_2$ (0.5 mL, 400 μM) into a solution of luciferase (0.5 mL, 400 μM) in standard buffer (0.05 M sodium P_i, pH 7.0, and 10 mM 2-mercaptoethanol) containing dodecanol (200 μM). Both solutions were at 0 °C. The reaction mixture was added to the top of a column of Sephadex G25SF (1 × 4 cm) preequilibrated with the above buffer mixture, contained in a Centrex Centrifugal Microfilter (Schleicher & Schuell, Keene, NH). It was then centrifuged (IEC clinical centrifuge) for 15 s (setting 4) until the solution had entered the gel. The same buffer mixture (1 mL) was then added to the top of the column and recentrifuged (15 s) and the eluted volume collected as the first fraction. The buffer addition was repeated until a fraction containing the maximum ratio of absorbance $A(370)/A(450)$ was obtained, corresponding to the intermediate II. Altogether, the preparation takes only about 5 min, and the free FMN is separated from the protein by 2–3 fractions. This procedure is similar to the one published by Tu (1986) but was developed independently. For the low-fluorescence form of the intermediate, the procedure is carried out with careful exclusion of room light. For the high-fluorescence form, the intermediate fraction is irradiated in a cool-white fluorescence light ring for about 5 min (0 °C), which us sufficient for developing the maximum fluorescence. If required, a concentrated stock solution of II was prepared by using an Amicon (Lexington, MA) ultrafiltration cell having a PM-30 membrane. The concentration of II was determined from the absorbance, $\epsilon(370) = 9000$ M^{-1} cm^{-1} (Tu, 1979).

Absorbance measurements were made with a Cary 14 spectrophotometer and fluorescence with an SLM 8000 spectrofluorometer. Correction of fluorescence spectra was made by reference to the absolute fluorescence spectral distribution of quinine sulfate (Velapoldi & Mielenz, 1980), and this was also used as the standard of fluorescence yield ($Q_f = 0.55$). Corrected excitation spectra were obtained by using the quantum counter method (Parker, 1968). The spectrofluorometer also was fitted with Glan–Thompson prisms in a T configuration for the measurement of steady-state fluorescence anisotropy (r). It was calibrated with a very dilute solution of glycogen ($r = 0.98$) and with FMN ($r < 0.02$). All fluorescence spectra were taken with polarizers in the "magic angle" positions to eliminate polarization effects (Lakowicz, 1983). Fluorescence and anisotropy spectra were taken with slit widths of 2 or 4 nm, except where indicated. Absolute bioluminescence intensities were measured with a photometer calibrated by the luminol chemiluminescence reactions (Lee & Seliger, 1965; O'Kane et al., 1986).

Light-scattering measurement of molecular weight was made with the SLM spectrofluorometer, with irradiation and detection wavelengths set at 600 nm, detection at 90° as for fluorescence, and 4-nm slits.

Dynamic fluorescence measurements were made with a laser excited system and single photon counting electronics. This type of apparatus is quite standard and is described in detail elsewhere (Brewer et al., 1987). The system consists of a mode-locked Nd/YAG laser, frequency doubled and synchronously pumping a dye laser, the output of which is intracavity dumped and again frequency doubled, to produce

single pulses of about 15-ps width and at a rate of 800 kHz or 4 MHz. A limitation of this type of apparatus is that a major effort is required to change the excitation wavelength more than about 20 nm, because it requires changing the dye, the associated optics, and the alignment. Three proprietary dyes from Exciton Chemical Co. (Dayton, OH) were used to provide the wavelength range of interest: R6G, 290–325 nm; DCM, 315–340 nm; LDS-751, 375–400 nm. Usually the average power of the excitation pulses was <1 mW. No irradiation damage of the samples would be expected at these power levels or could be observed. To help cut down stray radiation, glass color filters (Corning Glass Co., Corning, NY) were placed before the excitation of the sample and before the entrance slit of the emission monochromator. For λ_{ex} 300 nm, λ_{em} 340 nm, these were 7–54 before the sample and 7–59 on the emission monochromator, with entrance and exit slit widths both of 14 nm; for λ_{ex} 300–335 nm, λ_{em} 470 nm, 7–54 before and emission 5–57 or 3–73, with slit widths of 25 nm; and for λ_{ex} 385 nm, λ_{em} 470 nm, 4–96 before and emission 4–97 + 3–73, with slit widths of 25 nm.

The collection and analysis of data are also described elsewhere (Brewer et al., 1987; Kulinski et al., 1987). For the fluorescence decay model:

$$f(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

The reported parameters are from fits having reduced χ^2 <1.20 and a Durbin–Watson parameter (DW) greater than the value appropriate for the number of exponential terms (i) in the model: for $i = 1$, DW > 1.7; $i = 2$, DW > 1.75; $i = 3$, DW > 1.8. The same routines are used to fit the anisotropy decay data:

$$r(t) = \sum_i \beta_i \exp(-t/\phi_i) \quad (2)$$

but the procedure is not statistically rigorous (O'Connor & Phillips, 1984) because of incorrect weighting. Therefore, the fits are made to minimize χ^2 without the useful criterion that it be also close to unity. The DW test is also taken into account. Some data sets have been checked by using the more rigorous global procedures, and the final results were the same. One is a commercial routine from Applied Photophysics Ltd. (London, U.K.), and the other was provided by A.J.W.G. Visser (Vos et al., 1987).

Ultracentrifugation determination of the molecular weight of the high-fluorescence II was made by using the method of approach to equilibrium of Archibald (1947). Protein concentrations were monitored by absorbance. Since the preparations of the high-fluorescence intermediate also contained free FMN which absorbs at 370 nm, the net absorbance due to the intermediate was estimated by subtracting (0.96)A(450) from the total A(370). Typically, the net A(370) was in the range 0.2–0.3, i.e., 22–33 μM. Sedimentation was performed in an analytical ultracentrifuge at about 16000 rpm and 2 °C, using a precooled rotor. The cell was assembled and filled at 2 °C to avoid thermal decomposition of II. At 1–2-h intervals, the double-sectored cells were scanned at 370 and 450 nm to determine the net radial distribution of II. The data were analyzed by the method of Archibald (1947), and the molecular weight was estimated at the meniscus or cell bottom by eq 4 of that paper. The value of $\bar{v} = 0.73$ was calculated from the sequence amino acid composition of luciferase (Johnson et al., 1986).

Results

Figure 1 shows the fluorescence anisotropy spectra in the top panel and the absorption and fluorescence spectra in the lower panel of the high-fluorescence intermediate II. The absorption spectrum is very similar to the one reported by Hastings et al. (1973) for their preparation of intermediate II made by low-temperature chromatography. The corrected fluorescence spectrum has a maximum at 503 nm, and the fluorescence quantum yield of this sample is 0.30. On excitation of the sample at 460 nm, the fluorescence spectrum becomes identical with FMN (maximum at 538 nm; not shown). The total fluorescence spectrum is therefore a sum of two contributions, one from free FMN and the other from a species having a spectral maximum at shorter wavelength. This latter component may be separated from the total spectrum in Figure 1 by appropriate subtraction of an FMN fluorescence distribution (i.e., after correcting for excitation intensity at 360 nm). This procedure only shifts the maximum to 500 nm, so it is concluded that the major fluorescence is distinguishable from the bioluminescence spectrum which is also shown for comparison. Luciferase itself also has a small contribution to the fluorescence in this region, amounting to about 5% of the total fluorescence yield, but subtraction of this does not change the spectrum in Figure 1 significantly.

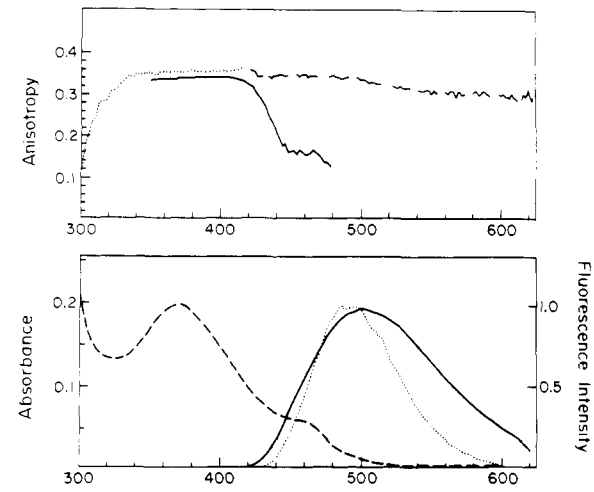

FIGURE 1: High-fluorescence form of intermediate II from *V. harveyi* luciferase (2 °C). (Top) Steady-state fluorescence anisotropy, for emission at 460 nm (···) and 490 nm (—) and for excitation at 370 nm (---). (Bottom) Absorption (---) and fluorescence (—; excitation 370 nm); in vivo bioluminescence (···) stimulated with tetradecanal.

The anisotropy spectrum scanning from 300 to about 420 nm is for fluorescence detected at 460 nm. This detection wavelength is chosen to ensure absolutely no contribution from FMN fluorescence. This steady-state anisotropy has a constant value of almost 0.35 in the range 340–420 nm, corresponding to the main absorption band in the lower panel. The solid line is the anisotropy scanned from 350 to 480 nm for the fluorescence set at 490 nm. Within the main absorption band, the anisotropy is lowered slightly due to the contribution of the small amount of free FMN fluorescence which has an anisotropy near zero. FMN bound to luciferase is not fluorescent (Baldwin et al., 1975). However, in the longer wavelength absorption band, maximum 460 nm, the anisotropy drops to 0.15. For the emission at 600 nm (not shown), the same behavior is observed: $r = 0.30$, 310–410 nm, dropping to 0.05, 440–500 nm.

These results are consistent with the longer wavelength part of the fluorescence being contributed to mainly by free FMN. In the preparation of the intermediate II, free FMN is separated by at least two fractions, so that the FMN in the intermediate fraction must be that initially bound to the luciferase and now dissociated (Baldwin et al., 1975; Vervoort et al., 1986b).

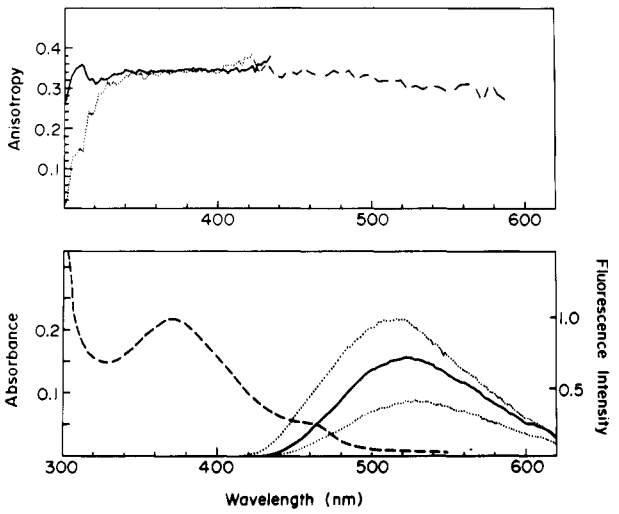

FIGURE 2: Low-fluorescence form of intermediate II from *V. harveyi* luciferase (2 °C; excitation slit 1 nm, emission slit 16 nm). (Top) Steady-state fluorescence anisotropy, for emission at 460 nm (···) at 490 nm (—) and for excitation at 360 nm (---). (Bottom) Absorption (---) and three fluorescence spectra (excitation 370 nm) showing the effect of irradiation, increasing the quantum yield and shifting the maximum to shorter wavelength. For the highest fluorescence spectrum, the emission slit was reduced to 8 nm.

The 490-nm emission has a corrected excitation spectrum showing no contribution from a band around 460 nm. This implies that the 460-nm absorption band is not a vibronic part of the main band but is an independent electronic transition. In Figure 1 (top), the anisotropy scan (dashes) from 420 to 620 nm is for the excitation wavelength fixed at 370 nm. The drop seen after 500 nm is due to the contribution to the fluorescence envelope of the free FMN. About a 10% decrease is seen by 530 nm, and since the free FMN has negligible anisotropy and about the same quantum yield of fluorescence as II (see below), a rough calculation estimates that about 10% of the absorbance at 370 nm is due to free FMN. Therefore, considering that there will also be some luciferase-bound FMN, the total absorbance at around 450 nm from total FMN could, within a factor of 2, account for the observed shoulder in the absorbance spectrum in Figure 1.

A further result from this anisotropy scan is that past 600 nm, a bioluminescence-like spectral distribution should make no contribution, and the fact that a significant anisotropy does remain is evidence that the intermediate II fluorescence extends to longer wavelengths than the bioluminescence spectrum.

Figure 2 is the spectral data for the low-fluorescence form of the intermediate II prepared under careful exclusion of room light. The absorption spectrum in the lower panel is identical with that in Figure 1 and with that published by Tu (1979) for the intermediate prepared with tetradecanol. The fluorescence quantum yield is about 0.03, and its fluorescence spectrum, the lowest of the curves to the right in the lower panel, is similar to that of free FMN, maximum 538 nm, except that it extends to lower wavelengths. Free FMN has no intensity at 470 nm. On continuous irradiation in the spectrofluorometer, the fluorescence blue-shifts, confirming the report of Balny and Hastings (1975), to become very similar to the spectrum of the high-fluorescence intermediate II. However, both the low-fluorescence form and that after irradiation exhibit the same high steady-state anisotropy, 0.34 (top panel, Figure 2), over the main absorption band, 330–400 nm. Below 320 nm, the anisotropy of the low-fluorescence form drops sharply as does the anisotropy of the high-fluorescence form (Figure 1). This can be attributed to absorption into the second electronic transition of the flavin intermediates. The failure of the irradiated sample to decrease its anisotropy in this region may be a scattering artifact but is otherwise unexplained.

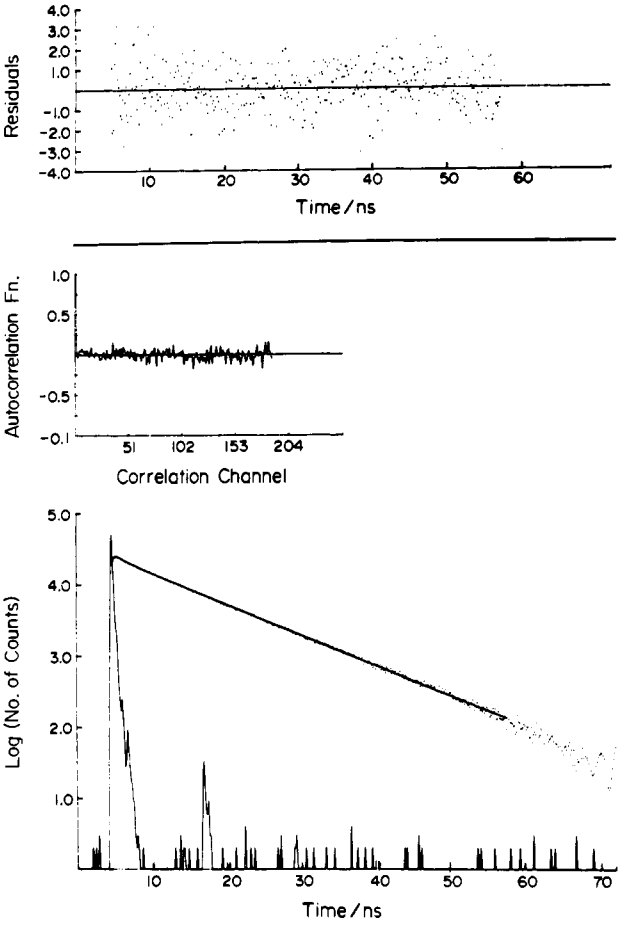

FIGURE 3: Total fluorescence decay of the high-fluorescence form of intermediate II (2 °C). The excitation pulse is the triangular shape at about 5 ns; the experimental data are fitted by the line 1.6 exp $(-t/10.0) + 0.28 \exp(-t/1.5) + 0.52 \exp(-t/0.48)$. In the top panels are shown the weighted residuals and autocorrelation function to verify the goodness of the fit. The statistical parameters are $\chi^2 = 1.12$ and Durbin–Watson parameter = 1.98.

In order to obtain the fundamental anisotropy (r_0) of the high-fluorescence intermediate, a Perrin plot was made (not shown). Ethylene glycol was added to increase the viscosity of the solution. From the intercept of the plot, $r_0 = 0.385$ (2 °C) for excitation at 370 nm and emission at 460 nm. The addition of ethylene glycol did not affect the fluorescence properties.

Figure 3 is the fluorescence decay of the high-fluorescence intermediate. The sharp triangular-shaped distribution centered at 5 ns is the laser excitation pulse. Its original width of about 15 ps is broadened by the detection system. The points are the fluorescence decay data, and the line is the best fit to a three-exponential function as indicated in the legend. There is a small oscillation in the data around 60 ns, an artifact generated by a routine used to remove some electronic interference from the cavity dumper driver (Brewer et al., 1987). The fraction of the total decay events, i.e., the total fluorescence, that are contributed by the 10-ns component (τ_1) is given by

$$\alpha_1\tau_1/\sum_i \alpha_i\tau_i = 0.96 \qquad (3)$$

The remaining fraction, 0.04, from the two faster decay components, would correspond closely to the luciferase con-

Table I: Emission Decay Parameters for Luciferase Intermediates II[a]

line	λ_{ex} (nm)	λ_{em} (nm)	fluorescence α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	anisotropy, ϕ (ns)	comments
1	300	450	63	10.1	9	2.9	31	0.21	68[b]	lines 1–16 are high-fluorescence form; lines 1–7 all have $A(370)$ ~0.2
2	310	470	80	10.0	3	1.4	17	0.34	87	
3	316	470	66	10.0	12	1.5	22	0.48	95	
4	316	470	71	10.1	8	2.2	21	0.49	93	
5	316	470	60	10.1	9	1.4	31	0.21	107	
6	323	470	78	10.2	12	2.7	10	0.30	86	
7	330	470	75	9.8	4	2.4	21	0.25	nd[c]	
8	385	470	64	10.2	6	0.7	30	0.08	90	$A(370)$ 0.95
9			70	10.1	8	1.9	23	0.3	93	av of lines 2–8
10			8	0.2	3	0.8	7	0.1	10	SD[d]
11	335	460	20	9.2	16	3.0	64	0.5	nd	no dodecanol
12	335	450	93	9.8	6	2.3	1	0.4		no dodecanol, 0.25 M P_i
13	320	500	74	10.2	4	3.7	22	0.7	91	$A(370)$ 0.1
14	316	470	43	10.0	10	2.0	47	0.30	114	$A(370)$ 0.03
15	316	470	28	10.0	20	2.4	52	0.30	nd	$A(370)$ 0.006
16	315	450	61	9.9	12	2.3	27	0.20	62	$A(370)$ 0.23, 10 °C
17	310	470	17	9.7	4	1.1	79	0.03	nd	lines 17–22, except 21, are low-fluorescence form; $A(370)$ 0.11
18	310	470	26	10.0	5	1.5	69	0.17	nd	$A(370)$ 0.17
19	310	470	24	10.0	5	1.2	71	0.12	94	$A(370)$ 0.33
20	310	470	28	9.8	10	1.5	62	0.08	nd	reaction mixture, not separated
21	310	470	38	9.8	6	1.5	57	0.07	87	this mixture irradiated until fluorescence increased 4×
22	335	460	23	9.0	20	3.3	57	0.4	nd	rxn mixture, no dodecanol

[a] All at 2 °C, except line 16; separation made in 50 mM P_i buffer, pH 7, containing 100 μM dodecanol. Amplitudes are normalized: $\alpha_1 + \alpha_2 + \alpha_3 = 100$. [b] Not included in the average, line 9. [c] nd, not determined. [d] SD, standard deviation.

tribution to the total fluorescence. It is therefore concluded that the high-fluorescence intermediate has nearly a single-exponential fluorescence decay. Substituting this 10-ns value into the Perrin equation, where ϕ is the rotational correlation time:

$$r_0 = r(1 + \tau/\phi) \quad (4)$$

then ϕ = 100 ns.

The ϕ is directly obtained from the decay of the emission anisotropy in Figure 4. A double-exponential fit to the data in Figure 4 gives ϕ = 100 ns for the major component, in good agreement with the above estimate. A single-exponential fit gives a slightly lower value, ϕ = 95 ns. The initial anisotropy, 0.35, is also consistent with that calculated from the r value in Figure 1, considering that the laser excitation is around 310 nm.

Table I contains the results of fluorescence and anisotropy decay analysis for the luciferase intermediate II, measured under a variety of conditions. Except as noted, the measurements in Table I were made on the separated intermediate stabilized by the presence in the solution of dodecanol, and at 2 °C. Lines 2–8 show that variations of the excitation and emission wavelengths do not affect the results, and therefore an average is given on line 9. For excitation at 300 nm, however, the anisotropy is small (Figure 1), and the decay data are very noisy; this ϕ = 68 ns has large uncertainty and is therefore not included in the average. Line 11 shows that if the intermediate is prepared without dodecanol, the long lifetime (~10 ns) is still evident, although of low amplitude because the intermediate is difficult to make in good yield without a stabilizing agent. Here and on some other lines, the correlation time was not determined (nd), either because the count rates were too low or for technical reasons. In line 12, the intermediate was stabilized by high P_i concentration. Again, the fluorescence decay is almost a single exponential since >98% of the decay events are found under the long lifetime. Dilution of the intermediate, lines 13–15, gives a lowering of the contribution of events having the long decay time compared to those having the shorter times, without affecting the magnitude of the lifetimes. At 10 °C (line 16), there is only a slight decrease in the contribution of the long-lived events, but as expected from eq 5, the ϕ is significantly lower than at 2 °C (line 9).

Lines 17–20 show that a three-exponential decay model is also required to fit the fluorescence decay from the low-fluorescence form of the intermediate, with a 10-ns component again making a dominant contribution. The rotational correlation time of the fluorophore is the same as for the high-fluorescence intermediate. Again, the same 10-ns fluorescence component is observed when the intermediate is generated in situ (lines 20 and 22), without any possibility of photochemical production of the high-fluorescence form by ambient light. The conclusion to be drawn from the results of Table I is that the fluorophore present in the preparations of both high-fluorescence intermediate II and low-fluorescence intermediate II is the same, differing only in its concentration.

Table II contains the results of analysis of the dynamic fluorescence properties of the fluorescent transient that occurs in the bioluminescence reaction. This species is generated by three methods: by the complete in vitro assay-type reaction, where $FMNH_2$ is added to a solution of luciferase and tetradecanal as described by Matheson and Lee (1983), or by the addition of tetradecanal to either form of intermediate II (Kurfuerst et al., 1984). For the complete reaction, several minutes are allowed to elapse after initiating the reaction before collecting the fluorescence data, to reduce the interference from the bioluminescence. The bioluminescence contributes a background to the fluorescence signal, but since this is uncorrelated, it is largely discriminated out by the signal collection logic.

It is evident from Table II that the fluorescence present in any of these reaction mixtures is again dominated by a 10-ns fluorescence component having a ϕ in excess of 95 ns.

The dynamic emission properties of native luciferase itself are presented in Table III, to serve here as controls. Each row is an average result from about five samples, with the exception

Table II: Emission Decay Parameters for the Fluorescent Transient in the Bioluminescence Reaction[a]

fluorescence						anisotropy		
α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	ϕ (ns)	SD (ns)	comments
50	9.8	20	2.6	30	0.3	118	17	complete reaction[b]
54	10.0	5	3	41	0.3	96		from high-fluorescence II[c]
46	10.4	10	4.6	36	0.2	96		from high-fluorescence II after 1 h
54	9.8	5	3	41	0.2	121		from high-fluorescence II after 2 h
40	10.2	12	2.4	48	0.2	127		from low-fluorescence II[d]
64	10.3	10	3.6	26	0.5	107		from low-fluorescence II after 30 min
58	10.3	8	2.4	40	0.2	113		from low-fluorescence II after 1 h

[a] 2 °C, with addition of tetradecanal. SD, standard deviation. [b] λ_{ex} 320 nm, λ_{em} 470 nm; luciferase $A(280)$ 13–16; $[FMNH_2]$ 80 μM (six samples; for τ_1, SD = 0.7). [c] λ_{ex} 340 nm, λ_{em} 490 nm; $A(370)$ 0.05. [d] λ_{ex} 320 nm, λ_{em} 470 nm; $A(370)$ 0.2.

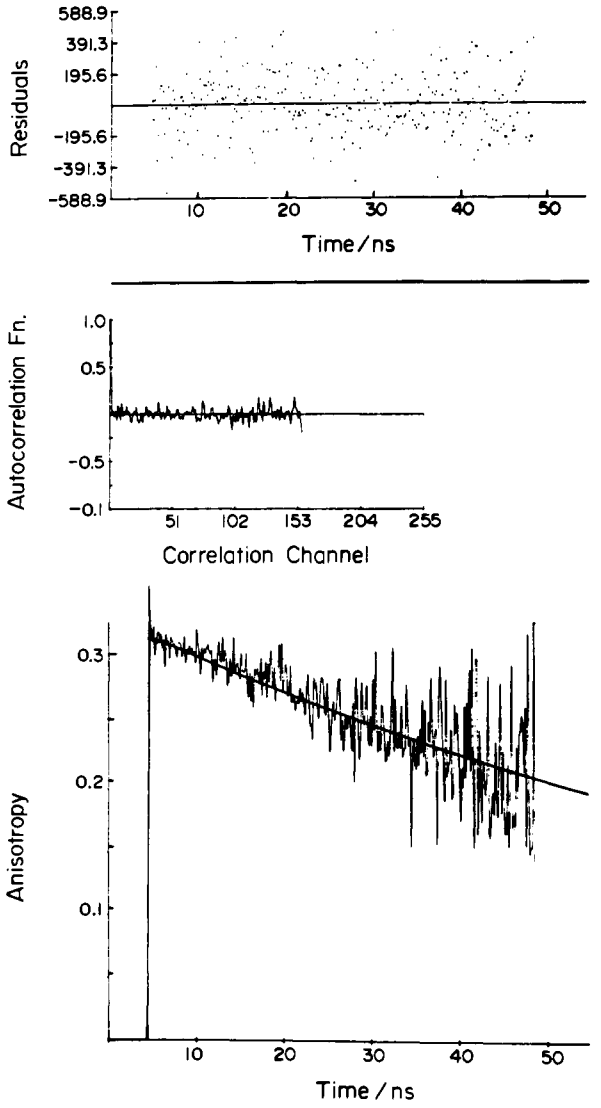

FIGURE 4: Decay of the fluorescence anisotropy of the high-fluorescence intermediate II (2 °C). The data are fitted by a double-exponential function (—): $0.3\exp(-t/0.05) + 0.11\exp(-t/101)$. The top panels show the relative residuals and autocorrelation to show that the line fits the data. The fit minimizes an appropriately weighted χ^2 function (O'Connor & Phillips, 1984), and the Durbin–Watson parameter is 1.99.

of rows 4 and 5. Both the intrinsic fluorescence from the eight Trp residues of the luciferase and the fluorescence from ANS, an extrinsic probe bound in a hydrophobic pocket of the luciferase, were recorded. For clarity, the standard deviation (SD) only of the longer correlation time is given, since this is most relevant to the present work. The minor contaminating chromophore in the luciferase itself makes negligible contribution to any of the other results.

A change in temperature from 22 to 2 °C causes an increase in ϕ of 2.0 times, accounted for by the 1.9 times increase in the η/T term in eq 5 where η is the solvent viscosity, M_r is

$$\phi = \eta M_r(\bar{v} + h)/RT \quad (5)$$

the molecular weight of the protein, $\bar{v}$ is the partial specific volume of the protein, h is the hydration of the protein, and T is the absolute temperature. The 2.0 experimental factor is independent of whether ϕ is obtained from the anisotropy decay of either the Trp residues' fluorescence or the bound ANS.

There is no change in the state of aggregation of luciferase with increase in concentration because ϕ is unaffected. Similarly, the fluorescence decay function is not dependent on concentration. On going from 22 to 2 °C for both intrinsic and bound ANS fluorescence, there is a small increase in the longest fluorescence lifetime and its amplitude, at the expense of the shortest lifetime component. This increase cannot be taken as evidence for structural change in the protein; more likely, it arises from the temperature dependence of some intramolecular quenching process. This problem will be reserved for another study as will be the wobble motion of the Trp residue as revealed by the fast component of the anisotropy decay (0.4 ns). Both the complex decay of the intrinsic fluorescence and this fast component of the anisotropy decay are typical of most proteins studied (Beechem & Brand, 1985). All the data are shown in Table III for completeness.

Tu et al. (1978) reported ϕ = 49 ns (23 °C) for ANS bound to luciferase and a single-exponential fluorescence decay function of τ_F = 14 ns. This τ_F = 14 ns is in agreement with the value in Table III for the longest component, but because their experiments were made with a flashlamp source, the extraction of ϕ has poor precision in this case, and therefore their 49-ns estimate is not to be regarded as significantly different from the value in Table III.

It has been shown previously (Visser et al., 1985) that the correlation time recovered from the anisotropy decay analysis is not dependent on the value of the fluorescence lifetime and that an accurate estimate of the correlation time can still be recovered when it is more than 5 times the magnitude of the fluorescence lifetime. As a further control, the ϕ for BSA monomer was obtained from the decay of the anisotropy of the intrinsic fluorescence: ϕ = 34 ns at 22 °C and 67 ns at 2 °C, for λ_{ex} 300 nm and λ_{em} 340 nm, for 1 mg/mL BSA in 50 mM P_i buffer. The longest fluorescence lifetime (2 °C) for the intrinsic fluorescence of BSA is also about 6 ns, as it is for luciferase. In the presence of ANS, ϕ = 70 ns (2 °C; λ_{ex} 300 nm, λ_{em} 340 nm), no significant change. The decay of BSA-bound ANS fluorescence anisotropy yields ϕ = 81 ns (2 °C; λ_{ex} 320 nm, λ_{em} 470 nm).

The correlation times obtained, therefore, for the native luciferase can be regarded as significantly lower than for the intermediate. Also, the 20% difference in the luciferase correlation times for the ANS fluorescence over the intrinsic

Table III: Emission Decay Parameters for Bacterial Luciferase

fluorescence						anisotropy				
α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	ϕ_1 (ns)	SD (ns)	β_2	ϕ_2 (ns)	comments
						Intrinsic Fluorescence[a]				
38	0.5	41	2.2	21	5.5	31	2	50	0.3	22 °C, A(280) 3.2–18
32	0.6	41	2.4	27	6.2	63	4	30	0.4	2 °C, A(280) 3.5
32	0.55	42	2.4	26	6.2	62	3	45	0.5	2 °C, A(280) 16
30	1.4	26	3.8	14	7.3	71		15	1	2 °C, A(280) 10,[b] α_4 = 30, τ_4 = 0.3
55	0.8	33	3.5	11	9	40		−26	0.06	2 °C, λ_{ex} 320 nm, λ_{em} 470 nm, A(280) 33.5[c]
						Bound ANS Fluorescence[d]				
19	0.8	21	5.2	60	14.8	38	4	20	0.3	22 °C, A(280) 16
20	0.5	14	4.2	66	15.7	77	7			2 °C, A(280) 3.5–4.2
13	0.9	17	6.2	70	17.1	72	7	40	0.3	2 °C, A(280) 16

[a] λ_{ex} 300 nm, λ_{em} 340 nm; $\beta_1 + \beta_2 = 100$. [b] High-fluorescence form of intermediate II, A(370) 0.26, A(280) 10 (one sample only; a four-exponential fit is required). [c] Contaminating fluorophore in the luciferase. [d] λ_{ex} 312 nm, λ_{em} 470 nm; ANS concentration was 0.5× the luciferase concentration.

fluorescence data can also be regarded as real. Table III also gives a measurement of the intrinsic fluorescence of a preparation of the high-fluorescence intermediate. The fluorescence decay is more complex than for the native luciferase, although the long component is still present but decreased in amplitude. The decay of intrinsic fluorescence yields a ϕ increased from native luciferase. Because only about 30% of the luciferase is converted to the intermediate form, this 71-ns value is an average from the luciferase in the native and intermediate states.

Measurement of the fluorescence quantum yield of the high-fluorescence intermediate was initially frustrated by a lack of reproducibility of results. The explanation is in Figure 5. The closed circles are the fluorescence intensity for diluted samples of the high-fluorescence intermediate. Whereas the absorbance does obey Beer's law (not shown), it is seen in Figure 5 that the fluorescence intensity is nonlinear with concentration. A log–log plot is made in order to fit in all the data. At the lowest concentration, the slope is about 2, a square dependence, changing to 1 at the high concentrations, a linear dependence. The open circles are the result of reirradiation of diluted samples. This fluorescence has a linear dependence (slope = 1).

A simple model to explain the nonlinear behavior of the fluorescence invokes protein complex formation with luciferase (E) catalyzed in the forward direction by light, e.g.

$$\text{II} + \text{E} \rightleftharpoons \text{II·E} \qquad (6)$$

where the complex II·E is responsible for the fluorescence. The solid line in Figure 5 is a quadratic solution for this equilibrium model assuming K = 10 μM, and the fit is good. Over the same absorption range, FMN (λ_{em} 538 nm) has a linear dependence of fluorescence intensity on concentration.

Molecular Weight Determination. Equation 6 predicts that at concentrations somewhat in excess of 10 μM, the high-fluorescence intermediate II will have a protein molecular weight of around 150 000. Three methods were used to determine the molecular weight of the protein in the intermediate II preparation—gel filtration, light scattering, and ultracentrifugation. In the first method, high-fluorescence II (0.05 mL) having A(370) = 7 was applied to an analytical HPLC column (TSK 3000; exclusion M_r 300 000 which was equilibrated and eluted (1 mL/min) with the dodecanol–buffer mixture. The maximum A(370) in the eluted fractions, at about 18 mL, had the same R_f as native luciferase, monitored by A(280). Also, the band shapes were the same. The R_f's were calibrated with standard proteins. The column dilution was ~30; nevertheless, the eluted intermediate would still have a concentration exceeding the 10 μM value assumed for the dissociation constant (eq 6). As a further control, a solution

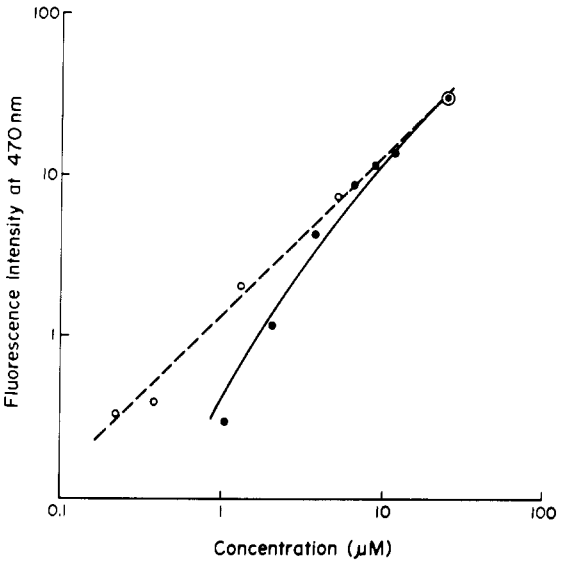

FIGURE 5: Fluorescence (470 nm, excited at 370 nm) of the high-fluorescence intermediate (2 °C; excitation slit 1 nm). The (●) data show that the fluorescence is not linear with dilution but obeys a quadratic function (—). The (○) data are the fluorescence after irradiating the diluted intermediate to a maximum fluorescence signal (2 °C, 5–10 min, excitation slit 16 nm). These latter data show a linear dependence on concentration.

of luciferase (400 μM) and lumazine protein (150 μM) in 0.1 M P_i buffer was chromatographed under the same conditions. The eluted fractions, monitored by A(405), also had the same R_f as native luciferase, but the band shape was twice as broad, accounted for by the known association of these two proteins (Visser & Lee, 1982).

The second method chosen was light scattering, and this suggested an increased molecular weight. The ratio of scattered (S) to incident (I) intensity of 600-nm light from a solution of protein of concentration C (in milligrams per milliliter) obeys the relation

$$S/I = bM_rC \qquad (7)$$

where b is a constant. Yeast alcohol dehydrogenase, BSA, and ovalbumin were found to obey eq 7, from which the value of the experimental constant b was derived. For the high-fluorescence intermediate of concentration in the range 25–100 μM, the value found for the molecular weight was 125 000, with a standard deviation of 35 000. This precision was not judged sufficient to conclude that the molecular weight of the luciferase was increased.

Table IV shows the results of determination of molecular weight by ultracentrifugation. As calibration standards, luciferase, BSA, and bovine IgG were sedimented under the

Table IV: Molecular Weight of Intermediate II by Ultracentrifugation[a]

sample	detection (nm)	method[b]	M_r ($\times 10^{-3}$)
BSA	280	M	74.1
IgG	280	M	153
luciferase	280	M	63.5
luciferase	280	B	72.4
luciferase	280	M	70.4
intermediate II	370[c]	M	66.4
intermediate II	370[c]	M	71.3
intermediate II[d]	450	E	78.1

[a] 2 °C, pH 7.0, 0.05 M P_i containing dodecanol, 200 μM; all samples of the intermediate were the high-fluorescence form. [b] Molecular weight was estimated by sedimentation equilibrium (E) or by the Archibald (1947) method of approach to equilibrium, measuring at the meniscus (M) or the cell bottom (B). [c] After subtraction of $(0.96)A_{(450)}$. [d] After decomposition to luciferase and FMN.

same conditions. This was done because of the imprecision of the Archibald method. Nevertheless, the literature values of the molecular weights were recovered. After the approach to equilibrium measurements were completed, the centrifugation was continued for 24–48 h until equilibrium was established. We conclude from these results that there is no evidence for an increase in molecular weight when luciferase forms intermediate II.

Discussion

The observations reported here raise two main question for discussion. Because these three fluorescent species occurring in the bioluminescence reaction have the same dynamic fluorescence properties, the first question is about the evidence that would distinguish these species from each other. The second is to explain why the rotational correlation time is so much greater for the fluorescent species than measured for native luciferase.

The steady-state spectral data show that the preparations of intermediate II contain four components. There is a minor fluorescent impurity in the luciferase which is responsible for the faster decay components of fluorescence detected at 470 nm. There is FMN, giving the shoulder in the absorption spectrum at 460 nm and the major contribution to the fluorescence spectrum of the preparation prior to its irradiation. The major components are the two forms of the intermediate flavin species; one is not, or only weakly, fluorescent, and the other is the efficiently fluorescent species having a fluorescence spectral maximum around 500 nm, with a fluorescence lifetime of 10 ns and a rotational correlation time of 100 ns at 2 °C. No change in the absorption maximum, 372 nm, occurs on photochemical conversion of the low- to the high-fluorescence form.

It is worth mentioning here that the spectral properties of the N(5) flavin adduct form of substrate-reduced lactate oxidase are very similar to those of high-fluorescence II. The structure of the flavin species is not known, but it is an unusual case of fluorescence from a reduced flavin. The fluorescence quantum yield is about 0.1, and the uncorrected fluorescence maximum is 476 nm (Ghisla, 1980). It has a dominant fluorescence lifetime of 10.8 ns and a high degree of emission anisotropy, 0.3 at 400 nm (22 °C; Visser et al., 1979). The absorption spectrum is the same in shape as Figure 1 minus the shoulder, with a maximum at 365 nm.

Kurfuerst et al. (1987) have presented data on a preparation of the FT partially separated from FMN and at pH 8.5. Increased stabilization of II at this pH was also shown by Gast (1978; Ziegler & Baldwin, 1981). The absorption spectrum showed a much greater FMN content in this FT preparation than found in II preparations. Nevertheless, they extracted the absorption spectrum of the FT, concluding that it had a shape very similar to II except with the maximum shifted about 10 nm to shorter wavelength. The reliability of this difference spectrum is lessened by the low values of the optical densities measured, the major contribution from the FMN in this region, and the uncertainty of the scattering background from the protein.

Equal reservation also needs to be applied to the fluorescence spectral distribution of the fluorescent transient because this also has to be obtained by subtraction of a large contribution from FMN (Matheson & Lee, 1983; Kurfuerst et al., 1984, 1987). The spectrum of the high-fluorescence II is distinguishable from the bioluminescence, but this difference is only brought out by measurement in the 600-nm region, where the signal is becoming small and, particularly in the case of the FT preparations, the FMN fluorescence is by far the major contributor. It is certainly feasible, however, that the binding of aldehyde to II could cause a blue-shift in the fluorescence of the flavin species to make it now indistinguishable from the bioluminescence (Matheson & Lee, 1983). However, it would be unusual to see a blue-shift in both absorption and fluorescence maxima; simple theory requires them to shift in opposite directions. The experimental situation remains that these small shifts are difficult to measure precisely and great caution must be applied to comparisons among these spectra.

The second major question for discussion concerns the interpretation of the 100-ns value obtained for the rotational correlation time of the fluorescent intermediates. The formation of a dimer (eq 6) with a calculated ϕ = 120 ns (eq 2, 2 °C, h = 0.35) would be consistent with the fluorescence intensity data of Figure 5. Attempts to measure this large increase in molecular weight do not provide positive support for this idea, although the results are not completely conclusive due to technical difficulties. The alternative explanation favored by the present evidence is that the luciferase undergoes a conformation change on going to the intermediates.

The observed ϕ = 100 ns is a remarkably high value for a protein of this size (Visser et al., 1979). Equation 5 predicts only 62 ns for a spherical rotator of M_r 77 000 (2 °C). In the first place, it means that the fluorophore must have little or no independent motion from the protein. From an NMR study, the same conclusion has been reached that FMN and $FMNH_2$ are also rigidly bound to luciferase (Vervoort et al., 1986b). Inflexibility of the binding environment would explain the fluorescence of the 4a-peroxyflavin intermediate. Model 4a-substituted flavins became fluorescent in a glassy matrix or when associated with some proteins, whereas they show no fluorescence in fluid solution (Ghisla, 1980).

The luciferase study was made to show that reasonable estimates of its rotational correalation time are obtainable from the anisotropy decay both of its intrinsic fluorescence and of the bound ANS fluorescence. Equation 5, which assumes a spherical shape, gives ϕ = 33 ns for luciferase at 22 °C in 50 mM P_i, in good agreement with the observations (Table III). The hydration of luciferase is calculated from its amino acid composition to be 0.35. A nearly spherical shape is consistent with the few hydrodynamic observations that are published (Visser & Lee, 1982; Holtzman & Baldwin, 1980). Since luciferase contains eight Trp residues, it would be expected that the intrinsic fluorescence anisotropy would average out over differing rotational axes, if any difference exists.

The difference in ϕ of about 15% between that from the decay of intrinsic fluorescence anisotropy and ANS can be rationalized in terms of the protein being a nonspherical rotor. Theory predicts that the observed value of ϕ may be greater,

or slightly less, than that calculated by eq 5, depending on the angles subtended by the transition dipoles and the axis of rotation of the protein (Ehrenberg & Rigler, 1972; Small & Isenberg, 1977; Barkley et al., 1981). The three-dimensional structure of luciferase shows an axial ratio of about 1.5 (Swanson et al., 1984). Assuming that in solution luciferase is a prolate ellipsoid of this same axial ratio, 1.5, the 20% higher ϕ for bound ANS can be accounted for from the tabulated calculations (Fleming, 1986), if its transition dipole lies along the longer axis. If in the intermediate form the luciferase were to undergo a conformational change to an ellipsoid with an axial ratio increase to about 2.2, this would be sufficient to account for ϕ = 100 ns, again assuming the transition dipole of the flavin species, in this case, lies along the long axis.

Barkley et al. (1981) provided experimental evidence for the influence of the relative orientation of the transition dipole and rotational diffusional axes on the observed average value of rotational correlation times. They compared the result from excitation into either the first or the second electronic transition, which should be about orthoganol to each other and therefore at quite different orientations to the principal diffusion axes of the macromolecule. However, for high-fluorescence II (Table I), excitation at 310 nm did not result in a significantly different value of ϕ than for excitation at 385 nm. The drop in steady-state anisotropy at the shorter wavelength indicates that excitation into the second transition must be occurring. Shorter wavelength excitation than 310 nm would complicate the interpretation due to excitation into the Trp residues, with energy transfer into the flavin fluorophore probably taking place. This negative result can again be rationalized from the tabulated calculations by assuming certain orientations.

Finally, to account for the fluorescence data of Figure 5, one proposal that can be made is that the photochemical conversion of low-fluorescence to high-fluorescence II is a photoreduction and the solution also becomes partially deaerated by the turnover of $FMNH_2$, so that on dilution reoxidation occurs back to the nonfluorescent oxidized form. Adequate model studies exhist (Ghisla, 1980) to suggest that this reduced species may be a 4a,5-dihydroflavin, but any further ideas about the chemical nature of these fluorescent intermediates of the luciferase reaction should await a more diagnostic experimental study. Structural characterization by NMR is planned.

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