Bioluminescence Spectral and Fluorescence Dynamics Study of the Interaction of Lumazine Protein with the Intermediates of Bacterial Luciferase Bioluminescence†

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ABSTRACT: The mechanism of the shifting of the bioluminescence spectrum from the reaction of bacterial luciferase by lumazine protein is investigated by methods of fluorescence dynamics. A metastable intermediate is produced on reaction of *Vibrio harveyi* luciferase with $FMNH_2$ and O_2. It has an absorption maximum at 374 nm and a rotational correlation time (ϕ) derived from the decay of its fluorescence (maximum 500 nm) anisotropy of 90 ns (2 °C). Lumazine protein from *Photobacterium phosphoreum* has an absorption maximum at 417 nm and a fluorescence maximum at 475 nm. Lumazine protein forms a protein–protein complex with luciferase, and the complex has a ϕ of ~100 ns. A mixture of lumazine protein and the intermediate would be expected to have an average correlation time (ϕ_{av}) around 100 ns, but instead, the result is anomalous. The ϕ_{av} is much lower and is also wavelength dependent. For excitation at 375 nm, which is mainly absorbed in the flavin chromophore of the intermediate, ϕ_{av} = 25 ns, but at 415 nm, mainly absorbed by the lumazine derivative ligand of lumazine protein, ϕ_{av} ~ 50 ns. It is proposed that protein–protein complexation occurs between lumazine protein and the luciferase intermediate and that in this complex energy transfer from the flavin to the lumazine is the predominant channel of anisotropy loss. A distance of 20 Å between the donor and acceptor is calculated. In the bioluminescence reaction of intermediate with tetradecanal, a fluorescent transient species is produced which is the bioluminescence emitter. If this species were the directly excited product, its emission rate (10^8 s^{-1}) is too fast for the excitation to be efficiently transferred (~2×10^7 s^{-1}) to the associated lumazine protein acting as a secondary acceptor. It is further shown that at high concentrations of the fluorescent transient, the bioluminescence spectral shift of lumazine protein is inhibited. The results are consistent with the sensitized chemiluminescence mechanism, wherein all these fluorophores present in the reaction compete for the source of the bioluminescence excitation.

Bacterial bioluminescence is produced by the reaction of bacterial luciferase with $FMNH_2$, O_2, and a long-chain aliphatic aldehyde, such as tetradecanal [for recent reviews, see Lee (1985) and Ziegler and Baldwin (1981)]. The bioluminescence emission has a broad spectral distribution with a maximum depending on the type of bacterium from which the luciferase was isolated. For example, the luciferase from *Vibrio harveyi* gives a maximum at 487 nm while that from *Photobacterium phosphoreum* is at 495 nm (Lee, 1982). Luciferase also reacts with $FMNH_2$ and O_2 alone to form a metastable species which can be reacted with aldehyde to produce the same bioluminescence as the complete reaction (Hastings & Gibson, 1963). This metastable species has been partially characterized by NMR as a 4a-substituted luciferase-bound flavin (Vervoort et al., 1986), and because it breaks down in the absence of aldehyde to FMN and H_2O_2, it has been proposed that the substitution is by a hydroperoxy group (Hastings et al., 1973; Hastings & Balny, 1975).

It has also been reasonably assumed that this species, named "intermediate II", is an intermediate of the bioluminescence reaction, and therefore its properties have been the subject of a number of investigations, mostly using the luciferase from *V. harveyi* because of the stability of its luciferase intermediate II (II).[1] The absorption spectrum of II is typical of a 4a,5-dihydroflavin, and it is weakly fluorescent, with an emission maximum around 500 nm (Hastings et al., 1973; Balny & Hastings, 1975; Lee et al., 1988). The fluorescence quantum yield is increased severalfold to 0.3 by irradiation with light (Balny & Hastings, 1975; Tu, 1979, 1986; Lee et al., 1988).

Another highly fluorescent species with spectral properties almost the same as this highly fluorescent form of II is formed in the course of the complete bioluminescence reaction (Matheson et al., 1981; Matheson & Lee, 1983) or on reaction of II with aldehyde (Kurfuerst et al., 1984). It is referred to as the "fluorescent transient", FT, because it has a lifetime of only about 30 min at 2 °C. Although the fluorescence spectral distribution of the FT cannot be obtained with good precision because it is mixed with the fluorescence of the product FMN, it has been concluded that it is the same as, or at least indistinguishable from, the bioluminescence spectral distribution (Matheson & Lee, 1983; Kurfuerst et al., 1984). Also, the fluorescence kinetics of FT are linked to the bioluminescence kinetics (Matheson & Lee, 1983). Therefore, it has been proposed that FT is the emitter of the bioluminescence in the reaction of luciferase.

It is known, however, that in *Photobacterium* in vivo, the bioluminescence emitter is a second protein called lumazine protein (Lee, 1985). This has been thoroughly characterized as a 21 000-Da protein to which is bound 6,7-dimethyl-8-ribityllumazine (Koka & Lee, 1979; Small et al., 1980; O'Kane & Lee, 1985a,b; O'Kane et al., 1985). The evidence for the emitter function of lumazine protein includes its effect on the

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[1] Abbreviations: II, luciferase intermediate II; HF, high fluorescence; LF, low fluorescence; FT, luciferase fluorescent transient; DW, Durbin–Watson parameter; τ, fluorescence lifetime; ϕ, correlation time; HPLC, high-pressure liquid chromatography; χ^2, reduced χ^2; SD, standard deviation.

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bioluminescence spectrum from the reaction of luciferase (Gast & Lee, 1978). The bioluminescence maximum in the reaction of the luciferase from *P. phosphoreum* or from *V. harveyi* is at 496 or 487 nm, respectively. If 10 μM lumazine protein is present, however, the maximum is now found at 480 nm for both types of luciferase. Also, with *P. phosphoreum* luciferase and lumazine protein at 30 μM concentration, the bioluminescence spectrum is the same as the fluorescence of lumazine protein itself, maximum 475 nm, as well as being the same as the in vivo spectrum (Lee, 1982; O'Kane et al., 1985a). Lumazine protein also changes the kinetics of the bioluminescence emission (O'Kane et al., 1985a; O'Kane & Lee, 1986).

The purpose of this present study was to test various proposals that have been made to explain how the emitters described above are excited by the exergonicity of the chemical reaction of luciferase. There is scant chemical information available on which to base a chemical mechanism as detailed as for some other bioluminescence systems (Gundermann & McCapra, 1987), so trying to clarify the physical process seems to be a profitable approach.

Matheson and Lee (1983) made a detailed kinetic study of the in vitro bioluminescence with *V. harveyi* luciferase and proposed that, in addition to the well-studied first-order bioluminescence, there was also a second-order process which they formulated as a sensitized chemiluminescence scheme:

$$\mathrm{II} + \mathrm{RCHO} \rightarrow \mathrm{EX} \quad (1)$$

$$\mathrm{EX} \rightleftharpoons \mathrm{FT} \rightarrow \mathrm{loss} \quad (2)$$

$$\mathrm{EX} + \mathrm{FT} \rightarrow \mathrm{P} + \mathrm{FT}^* \quad (3)$$

where EX stands for a chemically energetic species, e.g., a mixed peroxide, which breaks down to products, P, i.e., FMN, RCOOH, and H_2O, at the same time depositing the reaction exergonicity into the excited state of FT, labeled FT*. Their original scheme is simplified here, and the symbol are changed to facilitate discussion. Lumazine protein (LumP) then competes for this energy in another second-order process (Lee, 1982):

$$\mathrm{EX} + \mathrm{LumP} \rightarrow \mathrm{P} + \mathrm{LumP}^* \quad (4)$$

For lumazine protein to be able to affect the bioluminescence properties at concentrations only in the micromolar range, it was envisaged that a protein–protein complex of luciferase and lumazine protein should be formed (Ward, 1979). Although an association was demonstrated (Visser & Lee, 1982), it has not been possible to quantitatively account, for example, for the spectral shift in terms of the equilibrium parameters (Lee, 1985; Lee et al., 1989). The equilibrium dissociation constant is too large, especially for *P. phosphoreum* luciferase where it is in excess of 100 μM. The equilibrium with *V. harveyi* luciferase is also established slowly (Lee et al., 1989). An alternative idea is that lumazine protein associates strongly with intermediate II and to test this was the primary reason for embarking on this present study.

We report here that in a mixture of lumazine protein and II, the rate of decay of the emission anisotropy from the bound fluorophores is much faster than expected from just rotational relaxation of a spherical protein–protein complex of 100 kDa. This effect is attributed to an interchromophoric interaction, where excitation transfer is occurring between the flavin site on II within the protein complex and the lumazine group. A second finding is that lumazine protein is much less effective at shifting the bioluminescence from the reaction of the high-fluorescence form of II (HFII) or in the normal luciferase reaction if high concentrations of the FT are allowed to accumulate before the addition of lumazine protein. These results support the competitive model of bioluminescence of eq 3 and 4.

Materials and Methods

Bacterial luciferases were from *V. harveyi* strain MAVA, an aldehyde-deficient dark mutant, and from *P. phosphoreum* strain A13. The luciferases were purified to homogeneity and assayed for bioluminescence activity as described elsewhere (O'Kane et al., 1986). Lumazine protein was from *P. phosphoreum* strain A13 and was purified to spectroscopic homogeneity as described by O'Kane et al. (1985) except that the final step of purification used Blue–Sepharose chromatography rather than hydrophobic interaction HPLC as developed more recently (O'Kane & Lee, 1986). Protein concentrations were assayed by absorbance; for luciferases, ϵ(280 nm) = 85 000 M^{-1} cm^{-1}; for lumazine protein, the visible absorbance maximum is 417 nm, and ϵ(417 nm) = 10 100 M^{-1} cm^{-1} (O'Kane et al., 1985). All chemicals were the best grades that could be obtained commercially.

The preparation of both intermediates HFII and LFII was as described by Lee et al. (1988). For the measurement of its absorption spectrum, the fluorescent transient species (FT) was prepared by a similar procedure as follows. About 30 s after mixing the $FMNH_2$ (0.5 mL, 400 μM) with a solution of luciferase (0.5 mL, 400 μM) in 0.05 M P_i/10 mM 2-mercaptoethanol, and saturated with tetradecanal, at pH 7.0 and 0 °C, dodecanol was added to a final concentration of about 200 μM, and then the mixture was subjected to the filtration procedure with the Centrex Centrifugal Microfilter (Schleicher & Schuell, Keene, NH), as described for intermediate II. The dodecanol appeared to stabilize the FT for several hours, as measured by fluorescence (370 → 470 nm) or absorption (370 nm). Separation of the excess FMN product, however, was not as efficient as found for the chromatography of II.

Emission decay measurements were made with excitation from a laser system and detection by single-photon counting electronics (Lee et al., 1989). These techniques are described in that reference in detail. Steady-state fluorescence, absorption, and bioluminescence spectra were measured also as described before (Lee, 1982; Matheson & Lee, 1983). All spectra are fully corrected.

Data Analysis. The samples are excited with vertically polarized light, and the fluorescence is measured at 90° in the parallel or vertical direction, intensity I_v, and in the horizontal direction, intensity I_h. The time dependence of these two components is given by

$$3I_v = S(t)[1 + 2r(t)] \quad (5)$$

$$3I_h = S(t)[1 - r(t)] \quad (6)$$

where

$$S(t) = I_v + 2I_h \quad (7)$$

and

$$r(t) = D(t)/S(t) \quad (8)$$

where

$$D(t) = I_v - I_h \quad (9)$$

The function $r(t)$ is the fluorescence anisotropy. In fitting the data, the exponential decay models are assumed:

$$S(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (10)$$

$$r(t) = \sum_j \beta_j \exp(-t/\phi_j) \quad (11)$$

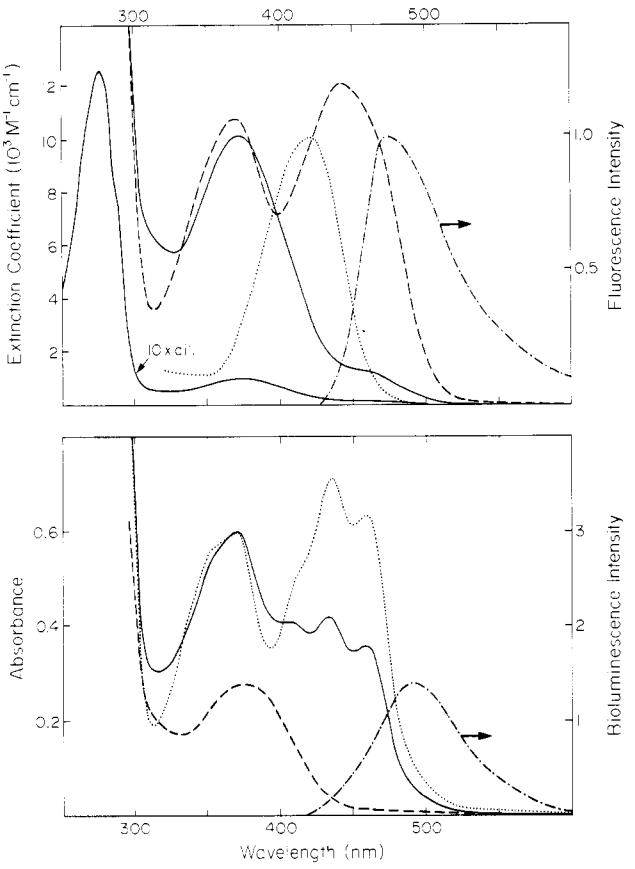

FIGURE 1: (Upper panel) Absorption spectrum of the high-fluorescence form of the luciferase intermediate II, 2 °C (solid line), and the product at 22 °C, after warming to 30 °C for 15 min (dashed line). The fluorescence spectrum of the intermediate II (excitation = 370 nm) is the dot–dash line with the arrow (Lee et al., 1988), and the absorption spectrum of lumazine protein (O'Kane et al., 1985) is the dotted curve, both at 2 °C. (Lower panel) Absorption of the fluorescent transient preparation, 2 °C (solid line), and after warming and recooling to 2 °C (dotted line). This product spectrum is scaled to the full-line absorbance at 460 nm and then subtracted from the full-line spectrum to yield a net (dashed line) corresponding to the fluorescent transient itself. The bioluminescence emission spectrum is the dot–dash line with the arrow (Matheson & Lee, 1983). All spectra are in 50 mM P_i, pH 7.0.

In most cases, $i = 2$ or 3, and $j = 1$ or 2.

Two independent data analysis methods were employed. All results presented are by using the method of Vos et al. (1987), and the application of this method is described in detail elsewhere (Lee et al., 1989). In this method, the $S(t)$ parameters are first derived by a minimized χ^2 fit to eq 10. With these parameters fixed, the exponential fit, eq 11, is then made to eq 8.

Some of the data were reanalyzed with a routine, "Lifetime", provided by Dr. Gary Holtom, University of Pennsylvania. In the Lifetime routine, all the fitting parameters are allowed to vary until a completely minimized χ^2 is obtained. It was found, however, that the results were not significantly different from the parameters derived from the method of Vos et al. (1987); i.e., the results did not differ by more than the sample to sample variation. An exception to this statement will be pointed out later in connection with Table IV.

Results

Absorption Spectra. The absorption spectra of HFII and of the FT are in Figure 1. The reference was the buffer solution eluted from the Microfilter column several fractions prior to the sample. The top panel shows the absorption spectrum of HFII (solid line) over a larger wavelength range than previously reported. Also shown is the spectrum of this sample at room temperature after it had been warmed (30 °C, 15 min) to convert II to the product FMN (dashed line). On the assumption that the concentration of the original II corresponds to that of the product FMN, the extinction coefficient of II is estimated as $\epsilon(374\text{ nm}) = 10\,300\ M^{-1}\ cm^{-1}$, in the range of the 9000–12 500 M^{-1} cm^{-1} values reported in the literature (Balny & Hastings, 1975; Tu, 1979).

We have found that excitation of fluorescence of II in the shoulder region of the absorbance spectrum around 460 nm results in a fluorescence spectrum which is identical with that of FMN (Lee et al., 1988). With the addition of steady-state fluorescence anisotropy data, we have concluded that this absorbance shoulder is mostly contributed to by contaminating FMN. The ratio $A(460)/A(374)$ is variable from preparation to preparation, but it is usually less than 20%. This contaminating FMN has not been subtracted in the absorption spectrum of II presented in Figure 1.

At 280 nm, both FMN and $FMNH_2$ have about the same ϵ, $\sim 30\,000\ M^{-1}\ cm^{-1}$. It is a reasonable assumption that II also has the same extinction coefficient at 280 nm because of its similar electronic structure. Then the total extinction at 280 nm for the preparation of II will include the contribution of the luciferase and the flavin chromophore, i.e., 30 000 + 85 000 M^{-1} cm^{-1}. Figure 1 shows the observed extinction at 280 nm for II is 135 000 M^{-1} cm^{-1}, and in view of the approximations, it can be concluded that the luciferase is quantitatively converted to II. In most of the preparations that will be used for measurement later, the total luciferase will be maintained in about 2-fold excess over that bound up in the intermediate II.

The lower panel of Figure 1 contains the absorption spectrum of FT. Both the total spectrum of the preparation (solid line) and that resulting from subtraction of the accompanying luciferase-bound FMN (dashed line) are shown. The FT is less stable than II, and as a result, larger concentrations of the FMN product occur in the course of the chromatography. Because of the affinity of FMN for luciferase, efficient separation of the two cannot be achieved by the rapid chromatography procedure.

The structure in the 400–450-nm region of the absorbance spectrum (Figure 1, lower panel, solid line) is characteristic of luciferase-bound FMN (Baldwin et al., 1975). The difference spectrum (dashed line), taken to represent that of the FT, is obtained by subtraction of the spectrum of FMN in the presence of the same concentration of luciferase and scaled at longer wavelength. This difference spectrum is observed to have a wavelength maximum 10 nm higher than the one published by Kurfuerst et al. (1987). This latter measurement was at a pH of 8.5, and the 10-nm difference in position of the absorption maxima could be due to a pK in this region. However, the present measurement is made at a 10 times higher absorbance for improved precision. It is concluded that, if both are measured at the same pH of 7, the absorption spectra of FT and II (Lee et al., 1988) are indistinguishable, with maxima at 374 nm. Also included in Figure 1 (upper panel), for the purpose of discussion later, are the absorption and fluorescence (right arrow) spectra of *P. phosphoreum* lumazine protein (Lee et al., 1985) and (lower panel) the fluorescence (right arrow) of the FT (Matheson & Lee, 1983).

Fluorescence Dynamics. Table I shows that the emission decay parameters of lumazine protein, either in the absence or in the presence of luciferase, do not depend on the excitation wavelength in the range 380–415 nm. For technical reasons, the measurements are restricted to this range. For this type

Table I: Dynamic Fluorescence Properties of Lumazine Protein (20 μM) in the Presence of Luciferase[a]

		anisotropy			fluorescence			
[E] (μM)	exn (nm)	β	ϕ (ns)	SD (ns)	α_1	τ_1 (ns)	α_2	τ_2 (ns)
0	415	0.36	23.6	1.5	0.96	14.8	0.04	3
0	398	0.33	24.9	0.6	0.92	14.7	0.08	1
0	380	0.34	25.8	0.9	0.93	14.7	0.07	4
50	415	0.32	52	3.3	0.95	14.3	0.05	6
50	380	0.34	57	1.4	0.92	14.2	0.08	5
100	398	0.36	84		0.88	13.8	0.12	1
			In 20% Glycerol					
0	390	0.32	56.4	0.1	0.94	14.3	0.06	3
50	390	0.31	82.1	2.5	0.91	13.9	0.11	3

[a] Conditions: 2 °C, 0.25 M potassium phosphate, pH 7.0; emission = 475 nm; $\beta = r_0$, the initial anisotropy; E = *V. harveyi* luciferase. The fluorescence anisotropy is fitted to $r(t) = \beta \exp(-t/\phi)$ and the fluorescence intensity to $I(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$. SD = standard deviation.

Table II: Dynamic Fluorescence Properties of Luciferase Intermediate II (High-Fluorescence Form)[a]

		anisotropy			fluorescence			
[II] (μM)	exn (nm)	β	ϕ (ns)	SD (ns)	α_1	τ_1 (ns)	α_2	τ_2 (ns)
50	415	0.35	83	1.2	0.83	10.2	0.17	0.8
100	398	0.29	74	2.5	0.93	10.3	0.07	1.4
50	385	0.33	75	2	0.88	10.3	0.12	1.4
50	378	0.34	84	6	0.86	10.2	0.14	1.1
			In 0.25 M P_i					
90	413	0.34	84		0.93	10.2	0.07	1.9
46	382	0.36	102		0.89	10.2	0.11	1.0
			In 20% Glycerol					
50	390	0.33	175	4	0.89	9.8	0.11	1.5

[a] Conditions: 2 °C, pH 7.0, 50 mM P_i, 160 μM dodecanol; emission = 475 nm; II = luciferase intermediate II.

of laser system, it is a nontrivial operation to alter the wavelength range substantially. The region 380–415 nm, however, provides a good sample of the lowest absorption band of lumazine protein. The increase of ϕ in the presence of luciferase is due to the formation of a protein–protein complex of increased molecular weight. There is no change in the lumazine protein absorption or fluorescence spectra on its association with luciferase (Lee, 1982; Visser & Lee, 1982). There is also a small decrease in τ_1 (~1 ns) in the presence of luciferase, as previously reported. It is shown elsewhere (Lee et al., 1989) that very similar results are found when the solution contains dodecanol and 50 mM P_i, in place of 0.25 M P_i. The Stokes–Einstein equation $\phi = M_r\eta(\bar{v} + h)/RT$, where $\bar{v}$ is the partial specific volume and h the hydration of the protein, shows that ϕ should be proportional to the solution viscosity, η. In 20% by volume of glycerol, the viscosity is increased by a factor of 2.4 at 2 °C. The increase in ϕ due to viscosity is seen in Table I to be as predicted (2.3) for lumazine protein. An increase of only 1.5 is found in the mixture with luciferase; evidently, the glycerol causes some dissociation of the protein–protein complex. Under the same conditions, luciferase containing bound ANS had a ϕ of 155 ns (SD 10 ns), an increase again of the expected factor, 2.3, from that without glycerol.

Each line in Tables I–III is an average of two to five separate samples. The sample standard deviation (SD) of ϕ is listed or the range in the cases of only two samples. The reproducibility is seen to be very satisfactory. The initial anisotropy (r_0) is calculated by interpolation in the data at a point corresponding to the excitation maximum which is taken to be $t = 0$. The first channel taken for the analysis was at $t = 150$ ps because, if the analysis was started at or before $t = 0$, convergence usually failed, or unrealistic parameters resulted. The fits are made to a minimum number of parameters based on the statistical criteria of a minimum reduced $\chi^2 < 1.2$, usually preferably close to 1.0, and a Durbin–Watson parameter (DW) greater than a certain minimum depending on the number of exponential terms (i or j) in the model: for $i = 1$, DW > 1.65; $i = 2$, DW > 1.75; $i = 3$, DW > 1.8 (O'Connor & Phillips, 1984).

Table II shows that there is no effect on change in excitation wavelength or of the stabilizing additives dodecanol or 0.25 M P_i on the emission decay parameters of HFII. The higher value of ϕ in the 0.25 M P_i solution is attributed to the slightly increased viscosity. The concentration of HFII was calculated from the $A(374)$. The half-life for the loss of bioluminescence activity of II was found to be variable. In some preparations, the activity was 90% depleted by the end of a series of emission decay measurements whereas, as measured by its fluorescence contribution, a considerable fraction of the original HFII was still present. Also, dodecanol is a more effective stabilizing agent for the bioluminescence activity than 0.25 M P_i. However, none of these variables seemed to make any difference to the emission decay parameters. In 20% glycerol, the ϕ is again increased by about 2 times.

The measurements in Tables I and II were made to act as controls to contrast with the results obtained for a mixture of lumazine protein and II (Tables III and IV). The correlation times (ϕ_{av}) in Table III are markedly smaller than expected for an average of the ϕ of II (Table II) and the luciferase and lumazine protein mixture of Table I. To calculate the expected value, we consider that, at 475 nm with a slit width of 10 nm used for the measurements in Tables III and IV, about 50% more of the lumazine protein fluorescence spectrum is sampled as compared with the fluorescence spectrum from II. Since the fluorescence quantum yield of lumazine protein is about twice that of HFII (Visser & Lee, 1980; Lee et al., 1988), then for equal absorbance of the two species, 3 times more fluorescence signal will come from the lumazine fluorophore than from the HFII fluorophore. At 415 nm, for HFII in the range 50–80 μM and lumazine protein at 20 μM, the absorbances are 0.20–0.32 and 0.20, respectively. From the cor-

Table III: Lumazine Protein Interaction with Luciferase Intermediates II: Dependence of Fluorescence Anisotropy Decay Parameters on the Excitation Wavelength[a]

exn (nm)	r_0	ϕ_{av} (ns)	SD (ns)	ϕ_1 (ns)	SD (ns)	β_1/r_0	SD
			(A) High-Fluorescence Form in Dodecanol				
415	0.33	38.9	0.4	24.1	0.5	0.62	0.04
400	0.33	35.8	4.2	22.4	1.4	0.66	0.17
385	0.32	28.9	3.1	22.4	2.5	0.80	0.2
375	0.32	24.8	0.4	20.9	0.2	0.89	0.14
			In Dodecanol and 20% Glycerol				
415	0.31	57.6	5.6	25.8	1.6	0.46	0.07
400	0.31	46.0	2.0	23.9	2.0	0.55	0.06
385	0.31	33.4	4.3	21.1	2.5	0.71	0.05
375	0.31	32.4	1.2	24.3	1.2	0.79	0.05
			In Dodecanol and 0.25 M P_i				
413	0.325	51.8	0.5	19.7	1.5	0.64	0.07
382	0.34	34.4	0.1	21.8	1.1	0.66	0.10
374	0.33	30.8	0.6	23.2	0.5	0.76	0.01
			(B) Low-Fluorescence Form in Dodecanol				
415	0.37	74	[b]				
398	0.32	34	[b]				

[a] Conditions: 2 °C, pH 7.0, 0.05 M P_i; emission = 475 nm; [lumazine protein] 20 μM; [intermediate II] 50–80 μM (from A_{374}); total luciferase concentration is 50 μM in excess of II. ϕ_{av} is derived from the model of fluorescence anisotropy decay, $r(t) = r_0 \exp(-t/\phi_{av})$, and ϕ_1 from the model $r(t) = \beta_1 \exp(-t/\phi_1) + \beta_2 \exp(-t/\phi_2)$ where ϕ_2 is constrained to a value of 150 ns in 20% glycerol, 100 ns otherwise, and $r_0 = \beta_1 + \beta_2$. [b] Only one sample measured.

Table IV: Lumazine Protein Interaction with Luciferase Intermediates II: Dependence of Fluorescence Intensity Decay Parameters on the Excitation Wavelength[a]

exn (nm)	α_1	τ_1 (ns)	SD (ns)	α_2	τ_2 (ns)	SD (ns)	α_3	τ_3 (ns)
			(A) High-Fluorescence Form in Dodecanol					
415	0.63	14.0	0.1	0.26	9.0	0.2	0.11	1.8
400	0.60	13.7	0.3	0.30	8.5	0.8	0.10	1.4
385	0.54	13.6	0.4	0.35	9.0	0.2	0.11	1.4
375	0.43	13.5	0.2	0.45	9.4	0.4	0.12	1.1
			In Dodecanol and 20% Glycerol					
415	0.67	13.7	0.2	0.24	7.3	1.5	0.09	1.6
400	0.63	13.3	0.2	0.25	7.4	0.6	0.12	1.2
385	0.55	13.2	0.3	0.34	8.3	0.8	0.11	1.2
375	0.51	12.9	0.3	0.38	7.7	1.2	0.11	1.2
375	0.38	13.7 fixed		0.50	9.3	0.3	0.12	1.1
			In Dodecanol and 0.25 M P_i					
413	0.67	13.8	0.2	0.25	8.7	0.5	0.08	2.2
382	0.54	12.9	0.2	0.35	8.7	0.4	0.11	1.1
374	0.55	12.6	0.2	0.34	8.5	0.4	0.11	1.3
			(B) Low-Fluorescence Form in Dodecanol					
415	0.80	13.0	[b]	0.14	5.7		0.06	1.0
398	0.70	12.5	[b]	0.15	6.4		0.15	1

[a] Conditions: 2 °C, pH 7.0, 0.05 M P_i; emission = 475 nm; [lumazine protein] 20 μM; [intermediate II] 50–80 μM (from A_{374}); total luciferase concentration is 50 μM in excess of II. [b] One sample only.

responding data in Tables I and II (lumazine protein–luciferase, 52 ns; HFII, 83 ns), the expected ϕ_{av} for this mixture is 60 ns or more. Instead, a value of 38.9 ns is found. For excitation at 375 nm, the calculation gives 76 ns, whereas the observed value is even lower, 24.8 ns.

The second striking result in Table III is that ϕ_{av} is not constant with excitation wavelength. Figure 2 shows, for example, the anisotropy decays of the same sample excited either at 415 or at 375 nm. The former decay was collected prior to the latter. The rates of anisotropy decay are clearly different. The wavelength effect is unchanged by increase of viscosity due to glycerol, or in the presence of both dodecanol and 0.25 M P_i. However, at each wavelength, 20% glycerol increases the ϕ_{av} only by (1.4 ± 0.1)-fold, to be compared with 2.4-fold calculated from the Stokes–Einstein equation. The slightly higher value of ϕ_{av} in the solution containing both dodecanol and 0.25 M P_i may be attributed to a greater degree of complexation of the lumazine protein with the luciferase (Table I; Lee et al., 1989). Finally, for the low-fluorescence form of II, the wavelength effect on the value of ϕ_{av} is even greater. The ϕ value of 74 ns at 415 nm is significantly greater than for the mixture with HFII (38.9 ns). At 415-nm excitation, almost all the fluorescence is coming from the lumazine ligand, and the fluorescence anisotropy only reflects the rotational diffusion of the luciferase-associated lumazine–protein complex. For complete complexation with luciferase, we have observed ϕ to be in the range 74–123 ns (Lee et al., 1989).

The right-hand side of Table III (ϕ_1) contains the results from fitting the anisotropy decay with a double-exponential function. It needs to be emphasized that statistical grounds alone do not demand a fitting function more complex than monoexponential, a common situation occurring in the analysis of anisotropy decay. If a fit of the data, such as in Figure 2, is made with the more rigorous "Lifetime" routine, where all the parameters of fluorescence intensity and anisotropy decay are allowed to vary independently, a fit with two correlation times (j = 2, eq 11) does provide a slight but significant reduction in χ^2, e.g., 1.3–1.2, as well as a more uniform distribution of the residuals. These results are not shown.

The main argument for using a complex anisotropy fit to these data, however, is the knowledge that there are three fluorophores determining the observed decay of fluorescence

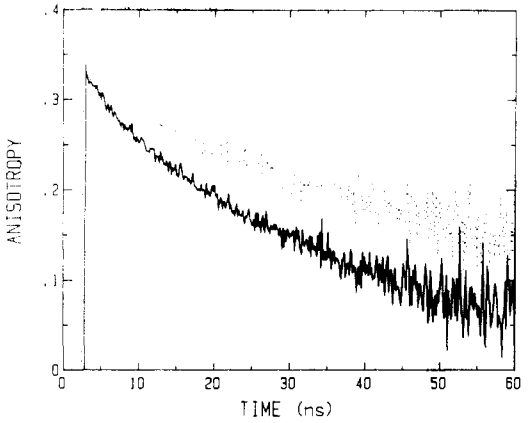

FIGURE 2: Decay of the fluorescence anisotropy of a mixture of lumazine protein (20 μM) and the high-fluorescence form of intermediate II (70 μM), at 2 °C in 50 mM P_i, containing dodecanol (150 μM). The fluorescence is detected at 475 nm. The upper curve is for excitation at 415 nm and the lower one for 375 nm.

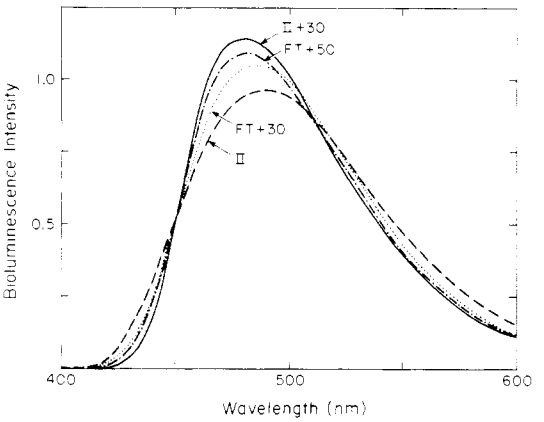

FIGURE 3: Inhibition of the lumazine protein shift of the bioluminescence spectrum by the fluorescent transient (FT) (2 °C). The reaction is initiated by adding tetradecanal to the low-fluorescence form of intermediate II (dashed line) or to II with lumazine protein (30 μM) (solid line). Alternatively, tetradecanal is added to II followed, after 30 s to allow maximum steady-state concentration of FT, by lumazine protein, 30 μM (dotted line) or 50 μM (dash–dot line). All are in 50 mM P_i, pH 7.0.

anisotropy. There is lumazine protein both free (ϕ = 24 ns) and complexed with luciferase ($\phi \sim$ 100 ns), and HFII (ϕ = 83 ns). The concentration of luciferase protein is in excess of 100 μM, so there will not be present very much of the unassociated lumazine protein, unless the luciferase in the HFII form has a much lower affinity for the lumazine protein than free luciferase. The two longer lifetimes can be lumped together because they are so close, and, in fact, the analysis is done with the longer correlation time, ϕ_2, constrained to a value of 100 ns. The constraint does not increase the χ^2 significantly over an unconstrained parameter fit, and setting ϕ_2 anywhere in the range 80–120 ns does not change the final result very much.

Table III lists the value of the freely varying components, ϕ_1, and its relative amplitude, β_1/r_0. Under all conditions measured, ϕ_1 remains relatively constant, only its amplitude changes. It is this amplitude change, therefore, that explains the decrease of the average, ϕ_{av}, as the excitation wavelength is moved from 415 to 375 nm. The value of ϕ_1 around 23 ns suggests that it could originate from free lumazine protein although the increase in its contribution at lower wavelengths would not be consistent with this proposal (Table I). In 20% glycerol, the ϕ for lumazine protein increases 2.3 times, to around 56 ns (Table I), yet in Table III, ϕ_1 is unaffected by this increase in viscosity and remains at 24 ns. In the case of the glycerol data, the analysis is made with ϕ_2 = 150 ns. A very similar result is observed for the mixture in 0.25 M P_i (ϕ_2 = 100 ns).

Table IV contains the fluorescence decay parameters for the same samples as in Table III. The origin of the two major lifetime components is readily apparent. The longest lifetime, τ_1 = 13.5 ns, is near the value measured for luciferase-associated lumazine protein (Table I). Consistent with this origin is the fact that its amplitude, α_1, decreases with shift of the excitation to shorter wavelength, as does the absorbance of lumazine protein (Figure 1). Concomitant with this decrease is a rise in the amplitude α_2, and since the absorbance of HFII also increases (Figure 1), τ_2 is assigned to HFII. The value of τ_2 appears 1–2 ns lower than for the HFII alone, however (10.2 ns, Table II). Both τ_1 and τ_2 remain relatively constant in these samples. Some caution must be made in the interpretation of such multicomponent fits, but the closeness of these lifetimes to those expected for the components of the mixture makes the assignments reasonable. On one line, the analysis is made with τ_1 constrained to 13.7 ns, the value known for the lumazine protein–luciferase complex, and the resulting value for τ_2 is still found lower than for HFII alone (Table II).

As indicated before, some of these data sets were also subjected to the method of analysis by the "Lifetime" routine, both where all parameters were allowed to be free or the case where the ϕ_2 was constrained. The final parameters were not significantly different from those listed in Tables III and IV, except for τ_2, which placed itself on the average around 7 ns. We conclude, therefore, that the lumazine protein causes some quenching of the HFII fluorescence lifetime. Consistent with this conclusion is the fact that the amplitude ratio, α_1/α_2, at 375 nm lies in the range 0.8–1.7, contrasting with the absorbance ratio of 0.14 in these samples between lumazine protein and HFII at this same wavelength (Figure 1). In other words, most of the photons are absorbed into HFII but are emitted at the longer lifetime, τ_1. This result would be equivalent to doing a steady-state excitation spectrum which is not feasible on these solutions because of the high absorbances employed.

Some of the data in Table III were also subjected to an analysis where one ϕ was associated with τ_1 and a second ϕ with τ_2. The result was not significantly different from that from the unassociated analysis, as might be expected, since the fluorescence lifetimes of the two fluorophores are of the same magnitude.

Bioluminescence Spectral Shifts. Figure 3 shows that the bioluminescence spectral shift induced by lumazine protein depends on the order of addition with respect to the aldehyde. The dashed line, labeled II, is the bioluminescence spectrum from reaction of LFII (5 μM) with tetradecanal. It is exactly the same as the spectrum from the reaction of MAVA luciferase and tetradecanal with $FMNH_2$. Both have a maximum at 492 nm, and little longer than the 487-nm value found previously using luciferase from the wild-type strain MAV (Lee, 1982). The solid line in Figure 3, labeled II + 30, is with 30 μM lumazine protein added to 5 μM II preparation, equilibrated for 30 s, and then tetradecanal added. The spectral maximum is now found at 479 nm, and, again, it is the same as found for the reaction starting with luciferase and tetradecanal and equilibrating with lumazine protein for 30 s before addition of $FMNH_2$.

Addition of tetradecanal to LFII (5 μM) produces the FT which reaches its maximum intensity at about 30 s (2 °C) (Matheson & Lee, 1983). At this point, about 50% of the total

bioluminescence photons have been emitted. The dotted line in Figure 3, labeled FT + 30, is the bioluminescence spectrum of this reaction following a 30-s equilibration time with lumazine protein (30 μM). The lumazine protein shift is clearly inhibited. The dot–dash line, labeled FT + 50, is with 50 μM lumazine protein, and a further shift of the spectrum to the blue results with a spectral maximum of 481 nm. In the MAVA reaction, this is the most shift that can be obtained. Starting with a higher concentration of LFII (20 μM) to generate a correspondingly higher concentration of FT, the inclusion of 30 μM lumazine protein hardly shifts the bioluminescence maximum from the 492-nm value (not shown). A similar series of results is found starting with a preparation of HFII under the same conditions. The lumazine protein shift is inhibited in an HFII-concentration dependent manner.

Discussion

The steady-state anisotropies of lumazine protein free or associated with luciferase (unpublished results), and of II (Lee et al., 1988), are constant over their long-wavelength absorption bands. These bands therefore consist of single electronic transitions, and this is also reflected in the constancy of ϕ (Tables I and II) within these absorption bands over the range of excitation wavelengths available from the dye laser used here. The dynamic fluorescence parameters are connected to the steady-state anisotropy (r) by the Perrin equation: $r_0 = r(1 + \phi/\tau)$. If there was no interaction of the two proteins in the mixture of lumazine protein and II, then a ϕ_{av} value of around 80 ns would be expected, and it would not depend on excitation wavelength. In the case of complexation between the two proteins without any communication between the two chromophores, an even higher value for ϕ_{av} should result, its actual value dependent of the axial ratio of the complex and the angles subtended by the transition dipoles and axes of rotational diffusion (Fleming, 1986).

The anomalously low value found for the correlation time of the mixture of II and LumP can be simply explained as due to energy transfer providing a route of anisotropy loss in addition to that from rotational relaxation. Excitation into the flavin chromophore absorption at 374 nm is transferred into the lumazine ligand within this lumazine protein–II complex. An alternative explanation that the appearance of ϕ_1, which causes the lowering of ϕ_{av}, is due to the presence of unassociated lumazine protein is eliminated on the grounds of the viscosity independence of ϕ_1 and the α ratios, as already discussed. A second possibility is that II and LumP form a tight complex in which an increase in segmental mobility occurs, reflected by the rate ϕ_1. An analogous situation is found in the case of BSA, where its well-characterized denaturation leading to separation of domains results in a lowered correlation time (Brewer et al., 1987). Again, such a segmental motion would not be expected to remain constant with increase in viscosity, nor is the α ratio with wavelength easy to rationalize on a segmental flexibility model.

Energy transfer between chromophores provides a route of anisotropy loss (Spencer & Weber, 1970; Dale & Eisinger, 1972; Kaminski et al., 1977). If the energy transfer is unidirectional from the flavin ligand on II to the lumazine, this would account for the fact that the amplitude of the presumed energy-transfer component, β_1, is higher for 375-nm excitation than for 415 nm. More of the excitation is absorbed by the flavin chromophore at the shorter wavelength than at the longer (Figure 1). At 415 nm, where most of the absorbed photons are in the lumazine chromophore, ϕ_{av} approaches that observed for the native luciferase–lumazine protein complex (Table I; Lee et al., 1989). The two values are quite comparable in the case of LFII–lumazine protein because of the negligible contribution of fluorescence from the intermediate II.

Figure 1 shows that the overlap between the fluorescence spectrum of II and the absorption of lumazine protein is very small. The overlap integral is only 1.5×10^{-16} M^{-1} cm^3, so that a dipolar energy-transfer mechanism would require the pair to be separated by much less than 20 Å for a better than 50% probability of energy transfer competing with the fluorescence decay, τ_1, of the donor. The Stokes radius of luciferase is 35 Å and of lumazine protein 23 Å, so that the complex must be very specifically aligned to place the two ligands in close proximity. If they are close enough for orbital overlap, of course then some other mechanism, e.g., of the exchange type, might be operating. No steady-state spectral evidence of orbital distortion is observed, however.

Bastiaens et al. (1988) have discussed the case of energy transfer between the bound cofactors FAD and FMN in cytochrome P-450 reductase. They obtained an equation for the time-dependent anisotropy decay which, simplified here by neglecting a term involving the rapid "wobbling in a cone" motion, is

$$r(t) = A_1 \exp(-2kt) + A_2 \exp(-t/\phi) \qquad (12)$$

where k is the rate of (dipolar) energy transfer. By comparison with the two-exponential fit to the data in Table III, we can identify ϕ_1 with the first term and estimate $k = 2 \times 10^7$ s^{-1}. This figure then enables a calculation of the average distance between the donor and acceptor, and, with the usual assumption of random orientation of the pairs leading to an orientation factor of 0.67 in the Foerster equation, the result is 20 Å.

Energy transfer is usually measured by the reduction in the fluorescence lifetime of a donor by the presence of an acceptor. This is not feasible in the lumazine protein–HFII system because both donor and acceptor species have overlapping fluorescence spectra of high yield and their fluorescence lifetimes are fairly close. The method of anisotropy decay measurement, however, has a clear advantage in that the two relevant decay parameters are well separated. Nevertheless, from the reduction of the fluorescence lifetime of HFII from 10.2 to about 8 ns in the presence of lumazine protein, a rough estimate of quenching rate is found to be 2.7×10^7 s^{-1}, consistent with the value derived from anisotropy decay.

The question now to be considered is whether this excitation transfer between the luciferase reaction site and the lumazine protein can explain the bioluminescence shifting effect. What is demonstrated is that excitation at the site of the chromophore of II can be transferred into excitation of the lumazine ligand. This transfer is occurring even when the flavin is not in its fluorescent form because (Table III) the LFII complex with lumazine protein also shows the short correlation time when excited at a shorter wavelength. It would be interesting to see if luciferase-bound $FMNH_2$ could also act as an excitation donor, because our fluorescence dynamics study of II indicated a considerable conformation change following oxygenation (Lee et al., 1988; Lee, 1988).

Kurfuerst et al. (1984) have proposed that the mechanism of bioluminescence emission from so-labeled "secondary emitters", such as lumazine protein, goes by excitation transfer from the FT species:

$$\text{II·LumP} + \text{RCHO} \rightarrow \text{FT*·LumP} \rightarrow \text{FT·LumP*} \qquad (13)$$

In this scheme, FT is proposed to be the primary excited product of the chemical reaction, emitting by its own fluorescence in the absence of added LumP:

$$II + RCHO \rightarrow FT^* \quad (14)$$

The FT species has spectroscopic properties which are very similar to HFII (Lee et al., 1988). Therefore, if FT is formed as a directly excited product of the bioluminescence chemistry, energy transfer to associated lumazine is feasible (eq 13). Since LFII does the same thing, however, then this means that a fluorescent donor species, e.g., FT itself, is not necessary for the excitation transfer. The absorption spectra of all these are the same, as also are their radiative lifetimes which, in the dipolar mechanism, is one term in determining the efficiency of energy transfer. Also in Figure 1, we see that both FT and HFII are as equally qualified as lumazine protein to be acceptor species, on the basis that they have about the same overlap of their absorbance spectrum with the (donor) bioluminescence spectrum.

The first problem with the eq 13 model is that the excitation transfer does not compete efficiently with the radiative rate from FT*, 2×10^7 s^{-1} versus 1×10^8 s^{-1}, respectively. The bioluminescence shifting results (Figure 3) also contradict this model. The more critical test of eq 14 here is that the presence of certain fluorophores in the reaction mixture, namely, HFII or the FT, inhibits the bioluminescence shift property of lumazine protein. If FT were the directly formed product (eq 14, then its presence according to the above scheme should not make *any difference* to the ability of lumazine protein to interact with the unreacted II, and a shift of the bioluminescence maximum should take place. On the other hand, the results are consistent with the scheme represented by eq 2–4.

The lumazine protein effects on the bioluminescence reaction are, in addition to the spectral shift, to increase the bioluminescence quantum yield and the rate of decay of light intensity. The quantum yield enhancement can simply be accounted for by the lumazine protein fluorescence quantum yield being 0.6 versus that of HFII or FT around 0.3. The kinetic effect means that lumazine protein catalyzes the chemical decomposition in a reactive encounter. It is a reasonable assumption that the IILumP complex mimics the structure of EXLumP, the reactive complex in which the bioluminescence excitation is deposited by a mechanism unknown, into the lumazine excited state. The proximity of the flavin reaction site and the lumazine ligand makes an electron-transfer process feasible for this excitation mechanism. Long-range electron transfer has been demonstrated in a number of cases, e.g., riboflavin binding protein (Farragi et al., 1985). This work also provides spectral evidence for the competition between the LumP and other fluorophores for excitation as originally formulated on kinetic evidence by Matheson and Lee (1983).

These bioluminescence effects also occur at lower concentrations of lumazine protein than the equilibrium constant for the protein complex with native luciferase. In these present experiments, the equilibrium constant for the *V. harveyi* luciferase–lumazine protein dissociation is 80 μM (2 °C, 50 mM P_i; Lee et al., 1989). At 5 μM luciferase and 30 μM lumazine protein, about 25% of the luciferase will be complexed. Figure 3 shows that for 5 μM LFII, much more of the bioluminescence is being emitted via the lumazine than can be accounted for by this proportion of the stable complex. The same result is found for the bioluminescence starting with luciferase. More critical is the observation that is outstanding, from previous work, that the interaction constant for the shift of the bioluminescence spectrum from *Photobacterium* luciferase by lumazine protein [3 μM (Lee, 1982)] is an order of magnitude smaller, and the equilibrium constant an order of magnitude larger [1 mM (Visser & Lee, 1982); 200 μM (Lee et al., 1989)], than for *V. harveyi* luciferase. Lumazine protein also causes marked kinetic changes in the *Photobacterium* bioluminescence (O'Kane et al., 1985; O'Kane & Lee, 1986). All these differences can be attributed to kinetic effects, and equilibrium association of the proteins involved, whether native luciferase or in the form of its intermediates, becomes irrelevant.

It is possible to use flavin analogues for generating II and to change the ligand attached to lumazine apoprotein (Mitchell & Hastings, 1969; Tu, 1982; Matheson et al., 1981; Kulinski et al., 1987). Therefore, variation of the spectral and associated properties of the donor and acceptor is an eminently feasible prospect, and the properties of such complexes should provide a deeper insight into the workings of this bioluminescence excitation-transfer process.

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How To Determine Protein Secondary Structure in Solution by Raman Spectroscopy: Practical Guide and Test Case DNase I

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ABSTRACT: For many proteins available in large (milligram) quantities, a three-dimensional structure determination by X-ray or NMR methods is very difficult, impossible, or too costly. In these cases, spectroscopic determination of secondary structure content can be a valuable source of partial information about protein structure in solution. In particular, Raman spectroscopy can be used to determine to fair accuracy the helix and sheet content of a globular protein. However, technical difficulties have hampered the routine application of the method: (1) The large background signal of aqueous solvent in the amide I region is difficult to subtract accurately. (2) The reference data set of Raman spectra of proteins with known crystal structure is incomplete, and the assignment of secondary structure in a known crystal structure is not unambiguous. (3) The mathematical problem of extracting structure information from the spectra is ill determined; i.e., there are many apparently satisfactory solutions for a given spectrum. We have now partly solved and partly sidestepped these problems by improving and simplifying existing methods. Here, we give a step-by-step outline of a procedure intended for routine determination of the percentage of α-helix and β-sheet from the amide I Raman spectra of proteins in solution. Its main features are (a) an uncomplicated procedure for solvent subtraction, aided by use of a divided spinning cell technique, (b) a numerically stable data handling algorithm, and (c) a clear statement of expected accuracy. In our hands, using the reference spectra of Williams (1983), helix content can be determined to an accuracy of 6 percentage points (largest error 12%) and β-sheet content to an accuracy of 5 percentage points (largest error 7%). However, the experimental distinction between parallel and antiparallel β-sheet does not appear possible without a significant expansion of the set of reference proteins. As a test we have measured the Raman spectrum of DNase I, a known structure treated as unknown, and derive 14% α-helix and 22% β-sheet content, compared to X-ray derived values of 20% helix and 25% sheet (hydrogen bonds per 100 residues). The error, −6% for helix and −3% for sheet content, is typical. The method can be a tool for checking the structural purity of genetically engineered proteins, detecting major structural alterations of mutant proteins, and providing a priori information as input to predictions of protein structure.

In Raman spectroscopy, incident photons scatter inelastically off the sample. In the amide I band of proteins the energy difference between incident and scattered beam corresponds to vibrational modes around 1660 cm^{-1} involving a few atoms primarily in peptide units (Carey, 1982), with a major component of C–O bond stretching. When peptide units are involved in secondary structure H-bonds, the normal modes are perturbed and the spectral lines shifted by different amounts for the different types of secondary structure. This fact is exploited empirically to derive information about the amount of secondary structure of proteins in solution. The method was developed by Garfinkel and Edsall (1958), Miyazawa (1960), Krimm and Bandekar (1986 and earlier papers), and Peticolas et al. (1979). An implementation by Thomas and

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