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Time-resolved fluorescence spectroscopy of lumazine protein from *Photobacterium phosphoreum* using synchrotron radiation

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Abstract. Time-resolved fluorescence on lumazine protein from *Photobacterium phosphoreum* was performed with synchrotron radiation as a source of continuously tunable excitation. The experiments yielded structural and dynamic details from which two aspects became apparent. From fluorescence anisotropy decay monitoring of lumazine fluorescence with different excitation wavelengths, the average correlation times were shown to change, which must indicate the presence of anisotropic motion of the protein. A similar study with 7-oxolumazine as the fluorescent ligand led to comparable results. The other remarkable observation dealt with the buildup of acceptor fluorescence, also observed with 7-oxolumazine although much less pronounced, which is caused by the finite energy transfer process between the single donor tryptophan and the energy accepting lumazine derivatives. Global analytical approaches in data analysis were used to yield realistic correlation times and reciprocal transfer rate constants. It was found that the tryptophan residue has a large motional freedom as also reported previously for this protein and for the related protein from *P. leiognathi* (Lee et al. 1985; Kulinski et al. 1987). The average distance between the tryptophan residue and the ligand donor-acceptor couple has been determined to be 2.7 nm for the same donor and two different acceptors.

Key words: Fluorescence decay, fluorescence anisotropy, anisotropic motion, energy transfer, orientation factor, bioluminescence, *Photobacterium*

Introduction

The structure and function of lumazine proteins from *Photobacterium* have now been well documented (Lee 1985). Lumazine protein functions as the light emitter of bioluminescence in *Photobacterium*, where in vitro the excitation energy is generated by the action of the enzyme luciferase involving reduced FMN, oxygen and a long chain aldehyde. Lumazine protein accepts the excitation energy from luciferase via a non-radiative mechanism and emits blue-green light by virtue of the strongly fluorescent, non-covalently bound prosthetic group 6,7-dimethyl-8-ribityllumazine (abbreviated as lumazine).

The protein lends itself perfectly to a thorough investigation using fluorescence techniques because it contains in addition to lumazine, a single tryptophan residue. Both natural fluorescent markers can be advantageously used to reveal dynamic avents as has been previously demonstrated (Lee et al. 1985; Kulinski et al. 1987). Since the protein is so well behaved it was decided to look in greater detail into several phenomena observed in our previous study of the lumazine protein from *P. leiognathi* (Kulinski et al. 1987). We previously employed mode-locked laser sources and time-correlated single photon counting to investigate time-resolved fluorescence of tryptophan and various ligands. Although not explicitly mentioned, the experiments were stretched over along period of time depending on the availability of the appropriate configuation of the laser (either argon ion laser lines or dye laser output in the UV). From the previous study an indication of anisotropic motion was obtained by comparing results obtained with different wavelengths of excitation and two different ligands. Also the rate of energy transfer between tryptophan (donor) and acceptor (either lumazine or 6-methyl-7-oxo-8-ribityllumazine, abbreviated as 7-oxolumazine) could be determined at one wavelength of excitation by monitoring the buildup of acceptor fluorescence.

It was therefore decided to continue the investigation with the lumazine protein from *P. phosphoreum* using tunable excitation from a synchrotron source. Employing synchrotron radiation we were able to completely confirm the previous results obtained with

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the *P. leiognathi* lumazine protein with the additional advantage of obtaining data within a relatively short experimental time. In fact, we could obtain unambiguous evidence for anisotropic motion of this protein, which is remarkable because of its low axial ratio (1.3) when assumed to be a prolate ellipsoid (O'Kane and Lee 1985a). Owing to global analysis of several sets of decay data (Knutson et al. 1983; Beechem et al. 1985), we could obtain good estimates of the correlation times involved and of the reciprocal rate constants for energy transfer between tryptophan and the two different lumazine derivatives. Also the agreement between these synchrotron data and results obtained with different laser set-ups turned out to be very good. A separate time-resolved fluorescence anisotropy experiment was conducted with the free lumazine in propylene glycol. The results provide clear evidence for anisotropic motion as has been observed in the past for other small fluorophores (Mantulin and Weber 1977; Barkley et al. 1981).

Materials

6,7-Dimethyl-8-ribityllumazine and 6-methyl-7-oxo-8-ribityllumazine were gifts of Dr. H. C. S. Wood (University of Strathclyde). The isolation of homogeneous lumazine protein from *P. phosphoreum* was carried out as described earlier (O'Kane et al. 1985). Lumazine protein was dissolved in a buffer of 50 m*M* sodium phosphate containing 1 m*M* mercaptoethanol at pH 7.0. The protein concentration was determined from published extinction coefficients (Lee et al. 1985) and amounted to 10 μ*M*. Apoprotein was prepared according to the protocol of O'Kane and Lee (1985b). Recombination with 7-oxolumazine was performed as described by Kulinski et al. (1987).

Methods

Fluorescence decay and fluorescence anisotropy decay measurements were performed using the Daresbury synchrotron radiation facility operating in single bunch mode (see Munro et al. (1985) for an extensive description of the time-resolved fluorescence set-up). Excitation wavelengths were varied between 440 and 260 nm at 10 nm intervals. The emission was detected with a microchannel plate photomultiplier (Hamamatsu R-1564-U) through interference filters: for lumazine fluorescence a Balzers 489 nm with a bandwidth 9 nm full width at half maximum (FWHM), for 7-oxolumazine fluorescence a Baird Atomic 402 nm (24 nm FWHM) and for tryptophan emission a Schott UV-DIL 339 nm (5 nm FWHM). The instrumental response function was obtained by tuning the excitation monochromator to the respective emission wavelength followed by measuring the scattering profile of a Ludox suspension. Several controls at selected wavelengths were taken with the laser set-ups at the affiliations of the authors. These instruments have been described in recent literature (Van Hoek and Visser 1985; Van Hoek et al. 1987; Visser et al. 1988; Lee et al. 1988).

All experiments involving proteins were carried out at 4 °C. The lumazine/propylene glycol experiments were conducted at 0 °C.

Data analysis was performed with the aid of computer programs developed by Vos et al. (1987). Global or simultaneous analysis of multiple decay patterns was carried out as outlined in the literature (Knutson et al. 1983; Beechem et al. 1985).

Theoretical background

Anisotropic rotation

After the pioneering work of F. Perrin (1934, 1936) the determination of anisotropic rotational diffusion of asymmetric molecular rotors with time-resolved polarized spectroscopy has received considerable attention (see, among others, Tao 1969; Belford et al. 1972; Chuang and Eisenthal 1972; Ehrenberg and Rigler 1972; Small and Isenberg 1977; Weber 1977; Barkley et al. 1981; Waldeck et al. 1981; Cross et al. 1983; Berkhout et al. 1984; Brand et al. 1985; Fleming 1986). We want to recall briefly the most important equations for the emission anisotropy of spheroidal proteins to place these in the context of lumazine protein as a prolate ellipsoidal rotor. Biopolymers such as proteins and aromatic molecules with bulky sidechains rotate subject to sticking hydrodynamic boundary conditions. Because of the surface roughness and of the hydrogen bonding capacity at the protein surface, solvent molecules are carried along during the rotation. These boundary conditions are appropriate for the highly charged lumazine protein (O'Kane and Lee 1985b) and for the reasonably polar lumazine molecule carrying a relatively large sugar residue and having a ground state dipole moment of 4 D (Platenkamp et al. 1987). For simple aromatic molecules slip boundary conditions prevail (Hu and Zwanzig 1974), as has been experimentally verified by both phase (Mantulin and Weber 1977) and pulse fluorimetry (Barkley et al. 1981).

The basic equation for the time-dependent fluorescence anisotropy of a fluorescent molecule rigidly attached to an ellipsoidally shaped protein is a triple exponential function (see e.g. Brand et al. 1985):

$$r(t) = \beta_1 \exp(-t/\phi_1) + \beta_2 \exp(-t/\phi_2) + \beta_3 \exp(-t/\phi_3) \quad (1)$$

where

$$\beta_1 = 0.1\,(3\cos^2\theta_A - 1)\,(3\cos^2\theta_E - 1) \quad (2a)$$

$$\beta_2 = 1.2\cos\theta_A \cos\theta_E (\cos\theta_{AE} - \cos\theta_A \cos\theta_E) \quad (2b)$$

$$\beta_3 = 0.3\,[2(\cos\theta_{AE} - \cos\theta_A \cos\theta_E)^2 - \sin^2\theta_A \sin^2\theta_E] \quad (2c)$$

θ_A and θ_E are the angles between the transition dipoles connected with absorption and emission, and the symmetry axis, respectively, and θ_{AE} is the angle between them. Note that β_1 is independent of θ_{AE}. The anisotropy at time zero, $r(0)$, is given by:

$$r(0) = \beta_1 + \beta_2 + \beta_3 = 0.2(3\cos^2\theta_{AE} - 1) \quad (3)$$

The correlation times ϕ are given by:

$$\phi_1 = (6\,D_\perp)^{-1} \quad (4a)$$

$$\phi_2 = (5\,D_\perp + D_{/\!/})^{-1} \quad (4b)$$

$$\phi_3 = (2\,D_\perp + 4\,D_{/\!/})^{-1} \quad (4c)$$

The diffusion coefficients for ellipsoids of revolution are:

$$D_\perp = 3/2\,D_s[\varrho\{(2\varrho^2 - 1)\,S' - \varrho\}/(\varrho^4 - 1)] \quad (5a)$$

$$D_{/\!/} = 3/2\,D_s\{\varrho(\varrho - S')/(\varrho^2 - 1)\} \quad (5b)$$

where ϱ is the axial ratio and D_s is the diffusion coefficient for a sphere of equal volume V, which can be equated by the Debye-Stokes-Einstein relation to the rotational diffusion coefficient:

$$D_s = kT/6\eta V \quad (6)$$

η is the viscosity, k the Boltzmann constant and T the temperature. For an oblate rotor $\varrho < 1$, while for a prolate rotor $\varrho > 1$; S' is then given by:

$$S'_{(\text{oblate})} = (1 - \varrho^2)^{-1/2} \tan^{-1}[(1 - \varrho^2)^{1/2}/\varrho] \quad (7a)$$

$$S'_{(\text{prolate})} = (\varrho^2 - 1)^{-1/2} \ln[\varrho + (\varrho^2 - 1)^{1/2}] \quad (7b)$$

Thus, knowing the axial ratio would reduce Eq. (1) to an expression in which the correlation times ϕ_1, ϕ_2 and ϕ_3 are reduced to an equivalent sphere correlation time ϕ_s. Fleming (1986) has tabulated the apparent correlation times for prolate and oblate rotors. As pointed out by Brand et al. (1985) all three pre-exponential factors can adopt a negative sign depending on the angular values involved (θ_A, θ_E, θ_{AE}, but also the angle between the projections of the transition moments in the equatorial plane). Not only this effect, but also lifetime and dynamic heterogeneity can lead to characteristic, sometimes peculiar, decay patterns as shown by simulations (Brand et al. 1985; Ludescher et al. 1987). Such an anomalous decay pattern has been observed experimentally for a highly elongated protein (Harvey and Cheung 1977). Lumazine protein has an axial ratio of approximately 1.3 (O'Kane and Lee 1985 a). The observed anisotropy therefore should not deviate significantly from a single exponential function, which can be seen when the tabulated values for the effective rotational diffusion relaxation times for a prolate ellipsoid of axial ratio 1.3 are substituted into Eq. (1) (Fleming 1986):

$$r(t) = \beta_1 \exp[-t/1.097\,\phi_s] + \beta_2 \exp[-t/1.061\,\phi_s] + \beta_3 \exp[-t/0.964\,\phi_s] \quad (8)$$

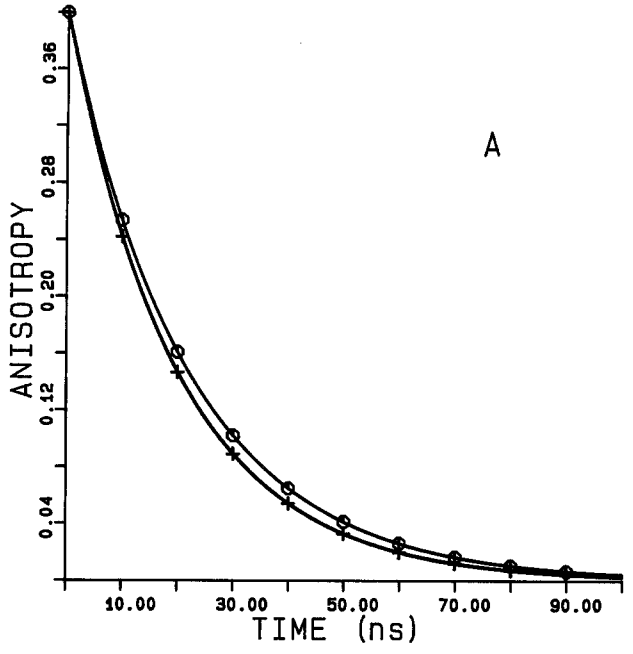
Up to now there is no crystal structure information on lumazine protein from which the location of the transition dipole directions relative to the diffusion principal axes could be inferred. Three optical transitions of 6,7,8-trimethyl-lumazine in the molecular frame have been determined by Sun et al. (1972). Analysis of the anisotropy decay would yield single correlation times which are effective averages of the true decay components. In order to obtain information from anisotropy experiments at different excitation wavelengths it is revealing to present some extreme cases, from which qualitative conclusions can be drawn (Fig. 1).

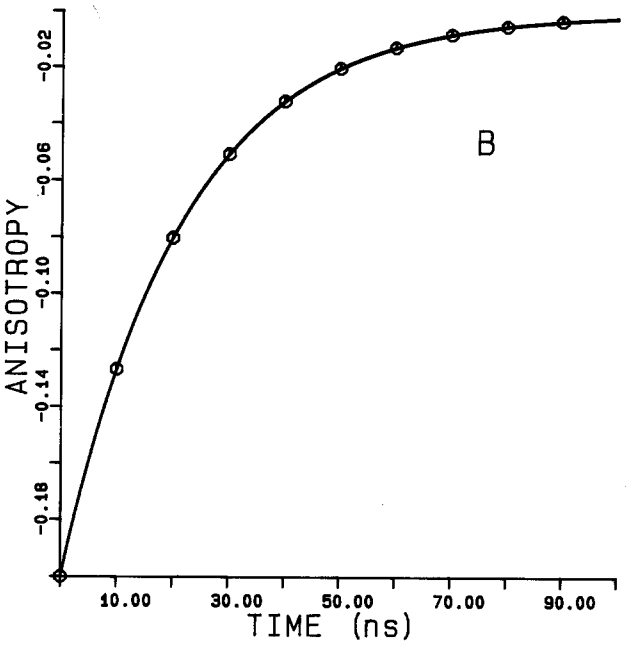
In Fig. 1 A three anisotropy decay curves are depicted assuming a spherical correlation time of 20 ns and a zero angle between absorption and emission moments. All decay curves start from $r(0) = 0.4$. The slowest decay is observed when the transition moment is parallel to the symmetry axis of the ellipsoid and gives rise to a mono-exponential decay. When the transition moment is perpendicular to the symmetry axis, the decay is bi-exponential, but cannot be distinguished from the exponential anisotropy decay of a sphere of equal volume. In Fig. 1 B two curves are presented for the case that the angle between absorption and emission moments is 90°. Both curves start at $r(0) = -0.2$. There is no apparent difference between the mono-exponential rise, when either absorption or emission moment is parallel to the symmetry axis, and the triple exponential rise, when either absorption or emission moment make an angle of 45° with the symmetry axis (and 90° with each other). In Fig. 1 C normalized anisotropy decays in semilog presentation are shown for the situation depicted in Fig. 1 B (45° with the symmetry axis) and for a sphere of equal volume. It is clear that the anisotropy for a spherical protein decays more rapidly.

Energy transfer

Tryptophan transfers excitation energy to lumazine via a weak dipole-dipole coupling mechanism (Förster 1965). The fluorescence lifetime of the tryptophan residue in lumazine protein will be shortened because of the presence of an extra decay channel with rate constant k_{DA} of energy transfer between donor D and acceptor A:

$$1/\tau_D = 1/\tau_{D_0} + k_{DA} \quad (9)$$





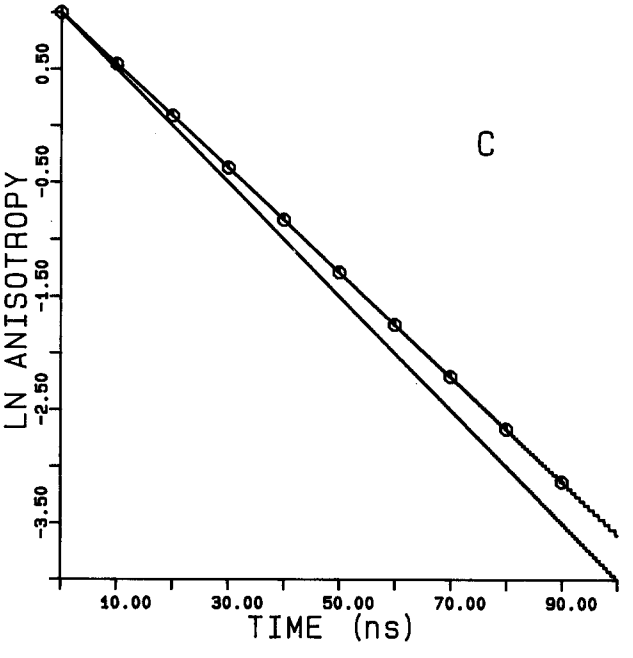


Fig. 1A–C. Simulations of fluorescence anisotropy decay profiles ($r(t)$) of a prolate ellipsoid with axial ratio of 1.3 assuming a spherical correlation time (ϕ_s) of 20 ns.
A The angle between absorption and emission transition moment (θ_{AE}) is zero: $-o-o-$: the angle between the transition moment and the main symmetry axis (θ_E) is zero: $r(t) = 0.4 \exp[-t/(1.097\phi_s)]$; the other curves overlap and are indicated with $-+-+-$: $\theta_E = 90$: $r(t) = 0.1\exp[-t/1.097\phi_s)] + 0.3\exp[-t/(0.964\phi_s)]$ and $r(t) = 0.4\exp(-t/\phi_s)$.
B $\theta_{AE} = 90$: $-o-o-$: θ_A (or θ_E) $= 0$, θ_E (or θ_A) $= 90$: $r(t) = -0.2\exp[-t/(1.097\phi_s)]$; ————: $\theta_A = \theta_E = 45$: $r(t) = 0.025\exp \cdot[-t/(1.097\phi_s)] - 0.3\exp[-t/(1.061\phi_s)] + 0.075\exp[-t/(0.964\phi_s)]$. Here also the two curves overlap.
C Semilog plots of the triple exponential function in B ($-o-o-$) and the spherical monoexponential decay of A (————)

τ_{D_0} is the fluorescence lifetime in the absence of A. k_{DA} is given by:

$$k_{DA} = (1/\tau_{D_0})(R_0/R)^6 \tag{10}$$

R is the distance between D and A and R_0 is the critical transfer distance given by:

$$R_0^6 = (8.8 \times 10^{-25}) Q_{D_0} \kappa^2 n^{-4} J \tag{11}$$

where J is the overlap integral, n refractive index of the surrounding medium, Q_{D_0} the quantum yield of donor fluorescence in the absence of A and κ^2 is the orientation factor. The measurable quantity is the transfer efficiency E:

$$E = 1 - Q_D/Q_{D_0} = 1 - \tau_D/\tau_{D_0} \tag{12}$$

where Q_D is the fluorescence quantum yield of D in the presence of A, τ_D and τ_{D_0} are the first order averaged lifetimes in case of multi-exponential fluorescence decay of D in the presence and absence of A, respectively (Kulinski et al. 1987). The transfer efficiency, on the other hand, is also equal to:

$$E = 1/\{1 + (R/R_0)^6\} \tag{13}$$

The fluorescence lifetime of the donor (τ_D) can also be determined by the buildup of acceptor fluorescence when the donor is excited (Kulinski et al. 1987):

$$F_A(t) \sim [(1/\tau_D) - (1/\tau_A)]^{-1} [\exp(-t/\tau_A) - \exp(-t/\tau_D)] + C\exp(-t/\tau_A) \tag{14}$$

in which also direct excitation of the acceptor with lifetime τ_A and constant C, depending on the experimental conditions, has been taken into account. In fact, in order to evaluate the rate constant of energy transfer, knowledge of the average fluorescence life-

time of the apoprotein (without acceptor) is still required. Note that the sign of the pre-exponential factors changes when $\tau_D > \tau_A$. However, in the case of lumazine protein we always have $\tau_A > \tau_D$. In order to determine the distance between donor and acceptor, it is very important to consider the possible values of κ^2 (Dale and Eisinger 1974; Dale et al. 1979). In lumazine protein we have a system in which the tryptophan donor has reorientational freedom and the lumazine acceptors rotate together with the protein. We need to know the angular range over which the tryptophan can move. An approximate expression, derived by Lipari and Szabo (1980), can be used:

$$r(t)/r(0) = (1 - A_\infty)\exp(-t/\phi_1) + A_\infty \exp(-t/\phi_2) \quad (15)$$

in which ϕ_2 is the rotational correlation time of the protein; ϕ_1 is a composite correlation time:

$$1/\phi_1 = 1/\phi_{\text{eff}} + 1/\phi_2 \quad (16)$$

where ϕ_{eff} is the effective time constant connected to the rapid reorientational motion. The pre-exponential factor A_∞ determines the angular range, over which the tryptophan can move and therefore, the order parameter S:

$$A_\infty = [1/2\cos\theta(1 + \cos\theta)]^2 = S^2 \quad (17)$$

Following Dale et al. (1979) the average, dynamic transfer depolarization factor $\langle d_T \rangle$ can be expressed as:

$$\langle d_T \rangle = \langle d_D{}^x \rangle \langle d_A{}^x \rangle (3/2\cos^2\theta_T - 1/2) \quad (18)$$

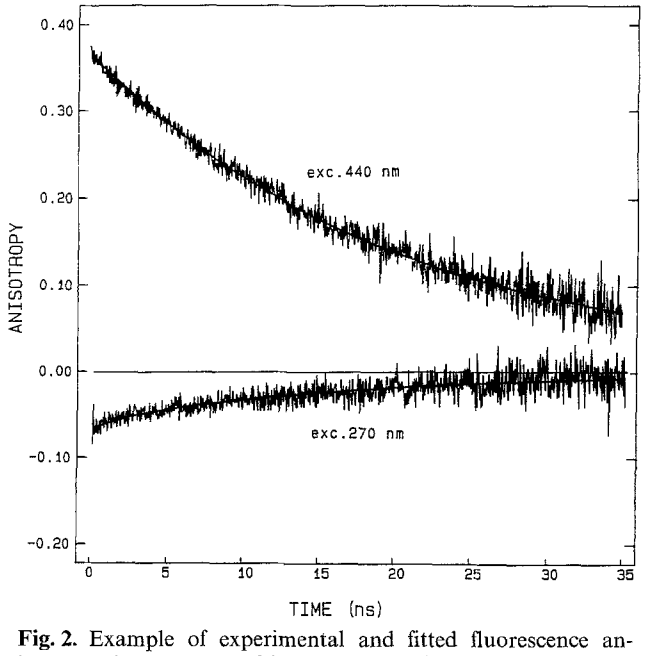


Fig. 2. Example of experimental and fitted fluorescence anisotropy decay curves of lumazine bound in lumazine protein from *P. phosphoreum*. The fluorescence was detected at 489 nm. Excitation at 440 nm: $\phi = 20.1 \pm 0.1$ ns. Excitation at 270 nm: $\phi = 17.0 \pm 0.2$ ns

$\langle d_D{}^x \rangle$ and $\langle d_A{}^x \rangle$ are the average, axial reorientation factors of donor and acceptor, respectively and θ_T is the angle between the symmetry axes of donor and acceptor distributions. In fact, the donor and acceptor depolarization factors can be determined from the fluorescence anisotropy decays Eqs. (15) and (16), while the transfer depolarization factor remains unknown. Dale et al. (1979) have developed contour plots to derive the boundaries of κ^2 given the axial reorientation factors of both *A* and *D*. The axial depolarization factors can be equated to the order parameters of the fluorescent molecule:

$$S = \langle d_{D,A}{}^x \rangle \quad (19)$$

This approximation is valid under the assumption that the transition dipole moment of either absorption or emission is oriented along the symmetry axis of an axially symmetric molecule. If the transition dipole moment is not oriented along the symmetry axis, this approximation also holds under the condition that the molecule is not spinning around its symmetry axis.

Results

Fluorescence anisotropy of lumazine as function of excitation wavelength

When lumazine protein is excited in the first electronic transition, the initial fluorescence anisotropy, $r(0)$, is high, approaching 0.36. The anisotropy decay as well as the fluorescence decay follow exponential patterns. When the excitation wavelength is varied and other electronic transitions are excited, the initial anisotropy becomes lower while the anisotropy decay profiles change as well, they remain exponential, however with different correlation times. When the excitation is below 300 nm, the initial anisotropy becomes negative. Two representative examples of anisotropy decay and their fits are given in Fig. 2. The fluorescence decay becomes clearly bi-exponential which will be discussed later. The complete situation is schematically represented in Fig. 3, in which both $r(0)$ and the correlation time are plotted as a function of the excitation wavelength. In Fig. 3 we have also indicated that the results obtained with two different computer programs are similar and lie within the 95% confidence interval (also indicated in Fig. 3). The values of $r(0)$, in particular, are identical. Two duplicate experiments, at high and low excitation wavelengths, are shown to illustrate the good reproducibility.

We can summarize the results presented in Fig. 3 as follows. In the range 370–440 nm the initial anisotropy is high, of the order 0.32–0.36. There is a gradual decrease in $r(0)$ below 370 nm, it becomes negative

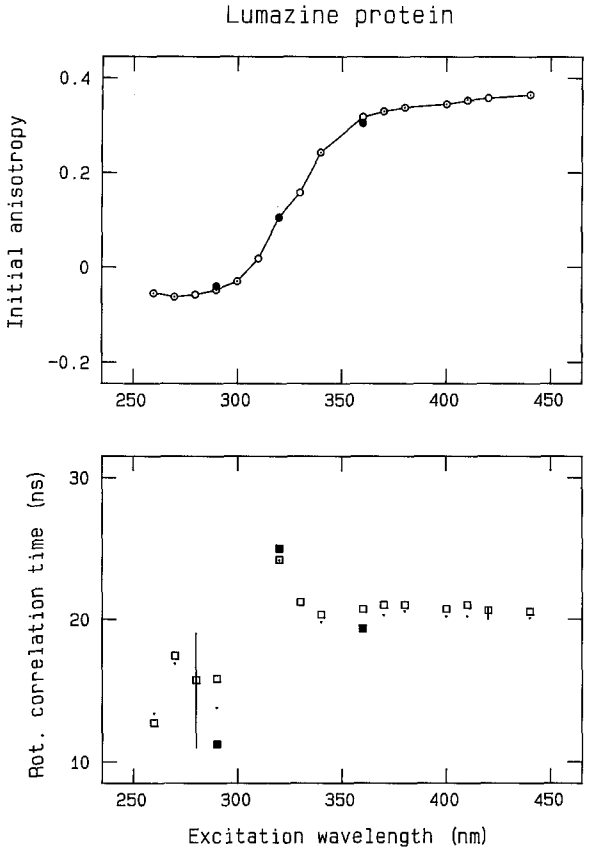


Fig. 3. Initial anisotropy ($r(0)$) and rotational correlation time (ϕ) of lumazine in lumazine protein from *P. phosphoreum* as a function of excitation wavelength. Analyses have been performed with the aid of two computer programs (see text). —o—o—: $r(0)$ and —□—□—: ϕ according to Vos et al. (1987); .....: $r(0)$ and ....: ϕ according to Beechem et al. (1985). At 280 and 420 nm 95% confidence intervals of the parameters have been indicated by the vertical line, for $r(0)$ the 95% confidence interval is equal to the thickness of the symbol. At 260, 320 and 360 nm duplicate measurements are indicated by solid symbols

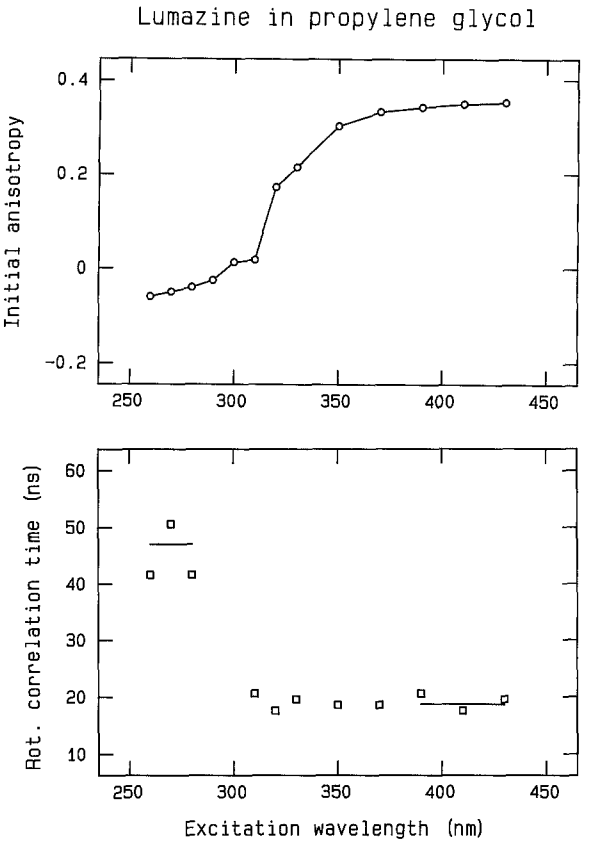


Fig. 4. Initial anisotropy ($r(0)$) and rotational correlation times (ϕ) of lumazine in propylene glycol as a function of excitation wavelength. —o—o—: $r(0)$ and —□—□—: ϕ; ———— global correlation times obtained by combining the data from these three excitation wavelengths

near 300 nm and it remains negative until 260 nm (the lowest between 260 and 280 nm, its value being around −0,06. The correlation time is more or less constant at 20–21 ns between excitation wavelengths of 350–440 nm. Below 340 nm there is a significant rise to about 24 ns (320 nm), followed by a sharp drop to approximately 16 ns in the excitation wavelength range 260–290 nm. Inspection of the decay patterns revealed that there is no oscillation in the anisotropy decay similar to that observed for perylene by Barkley et al. (1981).

Arguments will be provided to prove that energy transfer from tryptophan to lumazine contributes only slightly to the initial anisotropy of lumazine. First, lumazine itself dissolved in propylene glycol shows in general the same initial anisotropy-excitation wavelength pattern (see Fig. 4). Lumazine also displays anisotropic motion as revealed by the change in correlation time when the excitation wavelength is changed from the higher to the lower wavelength range. Interestingly, for the excitation range 260–290 nm the correlation times are at least twice as high as in the region of the first electronic excitation. This clearly shows that in lumazine protein the principal rotational diffusion axes are determined by the protein, while for free lumazine these axes must be dictated by the particular molecular shape. The second supporting argument results from the energy transfer experiments to be described in the next section.

Table 1. Initial anisotropies and rotational correlation times of 7-oxolumazine protein from *P. phosphoreum* as function of excitation wavelength

λ_{exc} (nm)	$r(0)$ (-)	ϕ (ns)
260	−0.005	–
270	−0.038	15.3±0.7
280	−0.070	13.8±0.4
290	−0.098	17.7±0.3
300	−0.003	–
330	0.323	20.8±0.2
360	0.338	19.4±0.2

We have also conducted similar experiments for lumazine apoprotein recombined with the closely related 7-oxolumazine. These experiments, summarized in Table 1, also indicate the presence of anisotropic protein motion.

Energy transfer from tryptophan to lumazine

Energy transfer from the single tryptophan residue to lumazine or 7-oxolumazine manifests itself in two ways. The first is the shorter average fluorescence lifetime of the tryptophan in the holoprotein as compared to the apoprotein. These results have been collected in Table 2. From the shortest average lifetime of the 7-oxolumazine protein it is evident that energy transfer from tryptophan to this ligand is most efficient. This is in accordance with the much larger overlap integral between the tryptophan fluorescence spectrum and the 7-oxolumazine absorption spectrum as presented in Fig. 5. In contrast to the rigidly attached lumazine chromophores, the single tryptophan residue exhibits a rapid reorientational motion. This is shown in Fig. 6 for the initial fluorescence anisotropy decay pattern of lumazine protein. All correlation times and amplitudes at excitation wavelength 300 nm have been collected in Table 2.

The second evidence for the occurrence of energy transfer between tryptophan and lumazine acceptors comes from the measurable rise of the lumazine fluorescence on excitation in the shorter wavelength region. Such an experiment is portrayed in Fig. 7 for lumazine protein, in which three curves are presented measured at 489 nm: the instrumental response function, a rapidly rising fluorescence for excitation at 440 nm and a more slowly rising fluorescence upon excitation at 300 nm. The fluorescence parameters at

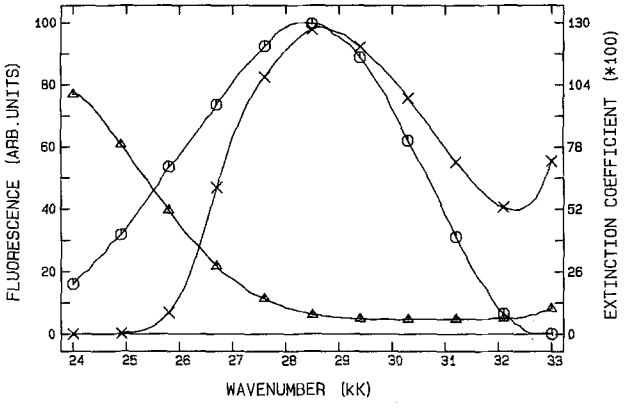


Fig. 5. Fluorescence spectrum of apoprotein (—o—o—) and absorption spectra of lumazine protein (—△—△—) and 7-oxolumazine protein (— × — × —) from *P. phosphoreum*. The overlap integrals for the two donor-acceptor couples were calculated as 4.47×10^{-15} cm^6/mol for the tryptophan-lumazine couple and 12.83×10^{-15} cm^6/mol for the tryptophan-7-oxolumazine couple

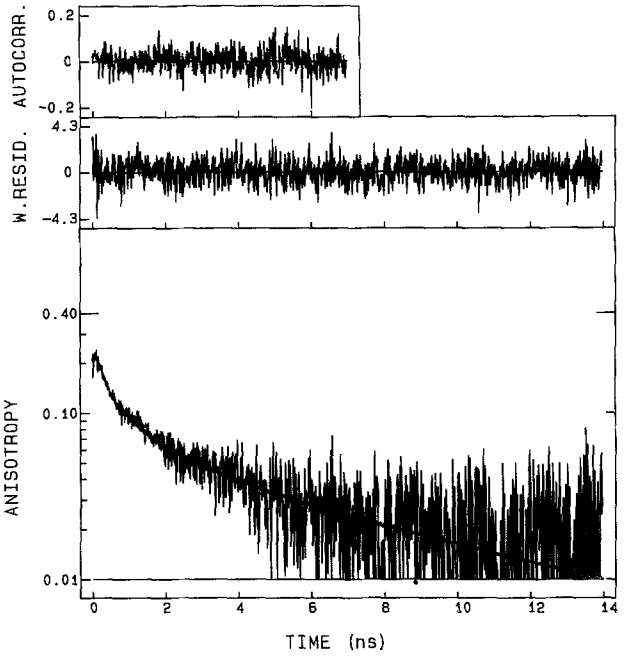


Fig. 6. Example of fluorescence anisotropy decay of the single tryptophan residue of lumazine apo-protein from *P. phosphoreum* and concomitant analysis. Only the first part of the decay is shown, together with the fitted function, weighted residuals and autocorrelation function of the residuals. For this experiment laser excitation at 300 nm was employed and fluorescence was monitored at 340 nm. The parameters are given in Table 2

Table 2. Fluorescence and anisotropy decay parameters of tryptophan in lumazine protein (LumP), 7-oxolumazine protein (7OlumP) and lumazine apo-protein (ApoP) from *P. phosphoreum*[a]

Sample	α_1 (–)	τ_1 ±0.05 (ns)	α_2 (–)	τ_2 ±0.1 (ns)	α_3 (–)	τ_3 ±0.05 (ns)	τ[b] (ns)	β_1 (–)	ϕ_1 ±0.02 (ns)	β_2 (–)	ϕ_2 ±0.5 (ns)
LumP	0.22	0.28	0.29	1.8	0.49	5.85	3.5	0.17	0.41	0.08	5.5
7OlumP	0.29	0.25	0.37	1.7	0.33	5.24	2.4	0.16	0.50	0.07	7.8
ApoP	0.19	0.33	0.24	2.2	0.57	6.03	4.0	0.16	0.39	0.09	5.1

[a] Excitation wavelength = 300 nm (laser); emission wavelength = 340 nm

[b] Average lifetime $\tau = \sum_{n=1}^{3} \alpha_i \tau_i$

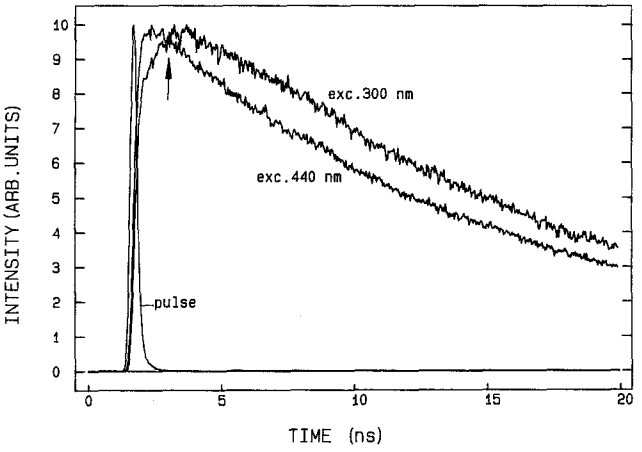


Fig. 7. Buildup of lumazine fluorescence in *P. phosphoreum* lumazine protein at 489 nm upon excitation at 300 nm as compared to direct excitation at 440 nm. The instrumental response function measured at 489 nm is also shown. The arrow indicates crossing of the two curves

Table 3. Fluorescence lifetimes of ligands in lumazine and 7-oxolumazine protein of *P. phosphoreum* as function of excitation wavelength[a]

λ_{exc} (nm)	α_1 (%)	τ_1 (±0.1) (ns)	α_2 (%)	τ_2 (±0.10) (ns)
Lumazine protein				
260	74	14.9	−26	0.23
270	77	14.9	−23	0.61
280	76	15.0	−24	0.60
290	78	15.0	−22	0.73
300	71	15.1	−29	0.97
340	100	14.9	–	–
380	100	14.6	–	–
420	100	14.8	–	–
7-oxolumazine protein				
260	76	7.3	−24	0.50
270	76	7.3	−24	0.72
280	79	7.3	−21	0.72
290	73	7.3	−27	0.12
300	95	7.2	− 5	0.50
330	100	7.2	–	–
360	100	7.2	–	–

[a] lumazine: emission wavelength = 489 nm; 7-oxolumazine: emission wavelength = 402 nm

different excitation wavelengths are collected in Table 3. Inspection of Table 3 (excitation at 290 and 300 nm) reveals that the short lifetime component, corresponding to the fluorescence rise is distinctly slower in the case of lumazine protein as compared to 7-oxolumazine protein, which is to be expected because of the less efficient energy transfer in the former. Further, it is seen that the lifetimes are not constant across the wavelength range of 260–300 nm, they tend to be shorter towards 260 nm. We suspect that this may be due to the involvement of other electronic states of tryptophan, so that transfer can take place more efficiently resulting in reduction of the short lifetime component. In the evaluation of the distances we will restrict ourselves therefore to data at 300 nm excitation, at which the ^1L_a transition is predominantly excited (Valeur and Weber 1977).

Another aspect concerns the relative contribution of direct excitation and excitation via energy transfer. In Table 3 the pre-exponential factors are scaled in such a way that the sum of the absolute values is equal to 100%. Thus the absolute values give directly the relative contribution of the excitation process. It is then immediately apparent from the data in Table 3 that direct excitation predominates over excitation via energy transfer.

Discussion

Anisotropic motion

From Fig. 3 it is evident that the correlation time of lumazine protein changes with the excitation wavelength, the correlation time being shorter in the low wavelength region. The presence of a maximum correlation time of 24 ns is to be noted. This effect strongly suggests that the protein behaves as an anisotropic rotor. The initial anisotropy as a function of excitation wavelength also shows characteristic changes, similar to that found by Sun et al. (1972) for a closely related lumazine derivative in ethanol at 77 K. From semi-empirical molecular orbital calculations three electronic transitions were predicted (Sun et al. 1972). The lowest electronic transition (400 nm) has a large oscillator strength, a second electronic transition with small oscillator strength is found at 300 nm and a third transition with large oscillator strength is located at 270 nm. The third transition moment makes an angle of 62° (measured counterclockwise) with the direction of the lowest optical transition, which is oriented from the nitrogen (3) towards the methyl group at carbon (7) (see Lee (1985) for structure and numbering). The second transition makes an angle of about 20° (clockwise) with the transition moment at lowest energy. The large angle of the third transition fully accounts for the negative initial anisotropy in the excitation wavelength region 260–290 nm, found both in the protein and in the model compound.

The simulated anisotropy decay patterns (Fig. 1) for a slightly elongated protein (axial ratio 1.3) are revealing, since they give a crude indication of the relative orientation of the transition moments with respect to the main symmetry axis of the protein. It is assumed for the purpose of discussion that the lowest

transition moment is not parallel to the symmetry axis, but makes a certain angle with it. It is tempting to assume that the second transition is oriented along the symmetry axis, because the longest correlation time is obtained upon excitation at 320 nm. Excitation in the 260–290 nm region gives rise to the shortest correlation time (16 ns), which must be very close to the correlation time of an equivalent sphere. The ratio of maximum and minimum correlation times ($\phi_{max}/\phi_{min} = 1.5$) must then correspond to a prolate ellipsoid of axial ratio 1.5 (Kulinski et al. 1987).

The different anisotropic rotation of free lumazine in propylene glycol is striking. Here excitation at 260–280 nm leads to much longer correlation times. This can be taken to indicate that this absorption moment is located along the molecular symmetry axis, giving rise to the situation sketched in Fig. 1C. The axial ratio, approximated by ϕ_{max}/ϕ_{min}, is about 2.5, which is significantly larger than for the protein.

This set of experiments demonstrates the usefulness of continuously tunable excitation wavelengths in the study of anisotropic protein rotation. It provides a good alternative for investigations of anisotropic protein rotation using the fluorescence of several internal and external reporter groups (Beechem et al. 1986). Although the axial ratio of lumazine protein is small, the effect is clearly demonstrated owing to the accuracy of the measured correlation times.

Energy transfer

Energy transfer from tryptophan to the lumazine acceptor is clearly demonstrated, as with the lumazine protein from *P. leiognathi*. The maximum and minimum values of the orientation factor can be estimated from the contour diagrams of Dale et al. (1979). The axial depolarization factor (or order parameter, see Eq. (19)) for tryptophan is similar for the three protein preparations (0.60 for the apoprotein, 0.57 for lumazine protein and 0.55 for 7-oxolumazine protein). There is no motion of the acceptor independent from the protein rotation, which yields an axial depolarization factor of unity. Since the transfer depolarization factor is unknown, only estimates of maximum and minimum values of the orientation factor can be provided (Table 4).

Some discrepancy arises when the two transfer efficiencies are evaluated. They differ significantly when they are determined either from the average fluorescence lifetimes of tryptophan in apo- and holoproteins or from the growing-in time constant of the acceptor fluorescence (Table 4). The short lifetime in the latter case leads to high efficiencies for both acceptors. Since there is hardly any difference in transfer efficiency for both donor-acceptor couples despite the large change in overlap integral, doubt is cast on the validity of these efficiencies. It is very well possible that the two closely spaced excited states of tryptophan are not equally active in the energy transfer process. Therefore the growing-in time constant representing the fluorescence lifetime of the donor in the presence of acceptor may be biased to the shorter lived component characteristic for the excited singlet state active in energy transfer. It is for this reason that the transfer efficiency is taken from the average donor fluorescence lifetimes in the absence and presence of acceptor molecules. It has been shown previously that the average lifetime is proportional to the quantum yield of the donor fluorescence enabling a reliable determination of the energy transfer efficiency according to Eq. (12) (Kulinski et al. 1987). Maximum and minimum distances between both donor-acceptor couples can then be easily retrieved (Table 4).

The minimum and maximum distances between the tryptophan residue and the two closely related acceptor molecules are within 0.1 nm identical, pointing to excellent agreement. The actual distance seems to be somewhat larger than that determined in the lumazine protein from *P. leiognathi* (Kulinski et al. 1987). The important conclusion is that the two chromophores are located at relatively large distance from each other bearing in mind that the Stokes radius is 2.2 nm (O'Kane and Lee 1985a). The difference between minimum and maximum distances is rather large because of the uncertainty in the orientation fac-

Table 4. Energy transfer parameters for donor-acceptor couples in lumazine protein from *P. phosphoreum*

Acceptor	E (–)	κ^2_{min} (–)	κ^2_{max} (–)	R_{0min} (nm)	R_{0max} (nm)	R_{min} (nm)	R_{max} (nm)
Lumazine	0.125[a] 0.76[b]	0.15	2.9	1.5	2.5	2.1	3.4
7-oxolumazine	0.40[a] 0.87[b]	0.15	2.9	1.9	3.1	2.0	3.3

[a] Energy transfer efficiency determined from Eq. (12) using the average lifetime as listed in Table 2 for apo- and holoproteins
[b] Energy transfer efficiency determined from Eq. (12) using the 'growing-in' lifetimes from Table 3 (excitation 300 nm) and the fluorescence lifetime of the apo-protein (Table 2)

tor as a consequence of the tryptophan mobility. On the other hand, the relatively large distance between the two fluorescent moleculares is in keeping with their location in two distinct domains of the protein. One domain containing the tryptophan residue is very mobile, while the other domain with the natural light emitter does not display any motion independent of the whole protein. The inflexibility of the ligand binding site might have a functional role in the sense that a microenvironment is provided for maximum light emission with no interference from dynamic quenchers in the lumazine vicinity. Confirmation of this distance information has to come from the tertiary structure of the protein.

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