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Analysis of time-resolved fluorescence anisotropy in lipid-protein systems

II. Application to tryptophan fluorescence of bacteriophage M13 coat protein incorporated in phospholipid bilayers

K. Peng [1]*, A. J. W. G. Visser [2], A. van Hoek [1], C. J. A. M. Wolfs [1], and M. A. Hemminga [1]

[1] Department of Molecular Physics and [2] Department of Biochemistry, Agricultural University, P.O. Box 8128, 6700 ET Wageningen, The Netherlands

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Abstract. The subnanosecond fluorescence and motional dynamics of the tryptophan residue in the bacteriophage M13 coat protein incorporated within pure dioleoylphosphatidylcholine (DOPC) as well as dioleoylphosphatidylcholine/dioleoylphosphatidylglycerol (DOPC/DOPG) and dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (DMPC/DMPG) bilayers (80/20 w/w) with various L/P ratios have been investigated. The fluorescence decay is decomposed into four components with lifetimes of about 0.5, 2.0, 4.5 and 10.0 ns, respectively. In pure DOPC and DOPC/DOPG lipid bilayers, above the phase transition temperature, the rotational diffusion of the protein molecules contributes to the depolarization and the anisotropy of tryptophan is fitted to a dual exponential function. The longer correlation time, describing the rotational diffusion of the whole protein, shortens with increasing temperature and decreasing protein aggregation number. In DMPC/DMPG lipid bilayers, below the phase transition, the rotational diffusion of the protein is slowed down such that the subnanosecond anisotropy decay of tryptophan in this system reflects only the segmental motion of the tryptophan residue. Because of a heterogeneous microenvironment, the anisotropy decay must be described by three exponentials with a constant term, containing a negative coefficient and a negative decay time constant. From such a decay, the tryptophan residue within the aggregate undergoes a more restricted motion than the one exposed to the lipids. At 20 °C, the order parameter of the transition moment of the isolated tryptophan is about 0.9 and that for the exposed one is about 0.5.

Key words: Subnanosecond fluorescence – Tryptophan – Bacteriophage M13 coat protein – Reconstituted lipid-protein systems – Motional dynamics

Abbreviations: DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; L/P ratio, phospholipid to coat protein molar ratio; $\langle\tau\rangle$, average fluorescence lifetime; $r(0)$, initial anisotropy; $r(\infty)$, residual anisotropy; S, order parameter of the transition moment of the tryptophan residue

* On leave of Shanghai Medical Equipment Research Institute, 77 Jiang Ning Rd. Shanghai, People's Republic of China
Offprint requests to: M. A. Hemminga

Introduction

The dynamic properties of viral proteins are of importance in understanding the mechanism of virus infection (Crowell and Lonberg-Holm 1986). A well known system used to study the infection of non-enveloped viruses is the bacteriophage M13-*Escherichia coli* system (Marvin and Wachtel 1975). When the virus enters the *E. coli* cell, it leaves its coat protein in the cytoplasmic membrane. It is assumed that after DNA duplication both the newly synthesized and the parental coat proteins are used for the assembly of new M13 particles (Wickner 1976). Details of the molecular mechanism of this assembly process are still unknown. To elucidate several aspects of the assembly process it is important to investigate the interactions between M13 coat proteins and lipid membranes, using different spectroscopic techniques to obtain information in various time windows (for a survey of various techniques applied to lipid-protein systems, see Devaux and Seigneuret (1985).

Time-resolved fluorescence spectroscopy has been widely used to investigate the events that occur in the time range from a few picoseconds to hundreds of nanoseconds (O'Connor and Phillips 1984; Van Hoek et al. 1987). With probes of sufficiently long excited-state lifetimes, the measurable range can even be extended to micro- or milli-seconds using phosphorescence spectroscopy. This optical technique is, therefore, very useful for studying the motions of the whole protein and of protein segments.

Many proteins contain amino acid residues, like tryptophan and tyrosine, which can function as intrinsic fluorescent markers to detect protein dynamics without perturbing the biosystem to be studied (for a review, see Beechem and Brand 1985). However, because of the limit-

ed excited-state lifetime of tryptophan, the measurement of mobility is restricted to the (sub)nanosecond time domain. Subnanosecond resolved anisotropy of tryptophan fluorescence has been used to investigate the motion of the tryptophan residues in a wide variety of proteins, such as nuclease B (Munro et al. 1979), adrenocorticotropin (Ross et al. 1981 a), horse liver alcohol dehydrogenase (Ross et al. 1981 b), myoglobin (Hochstrasser and Negus 1984), porcine pancreatic phospholipase A2 (Ludescher et al. 1985), human serum albumin (Vos et al. 1987), and ribonuclase T1 (Chen et al. 1987).

A single tryptophan is contained in the bacteriophage M13 coat protein (Nakashima and Koningsberg 1974), enabling the investigation of interactions between the protein and the membrane without using an extrinsic probe. In membranes the protein tends to aggregate (Wickner 1975; Spatz and Strittmatter 1971; Datema et al. 1987a and 1988) with a concomitant change in conformation (Nozaki et al. 1976; Wilson and Dahlquist 1985; Datema et al. 1987a). The motion of the entire protein will be dependent on the aggregation state (Cherry 1979). The variation in conformation of a protein in different aggregation states may result in a variety of local fluorescence lifetimes and/or local anisotropies. As a result of the combination of the motions of the entire protein and the tryptophan residue in different environments, which, in general, occur in different time scales, the time-resolved anisotropy decay is complicated.

Using time-correlated single photon counting, the subnanosecond fluorescence of tryptophan in the bacteriophage M13 coat protein incorporated into micelles and synthetic DMPC/DMPA bilayers has been previously measured in our laboratory, to study the effect of the membrane on the mobility of the tryptophan residue (Datema et al. 1987a). In the present paper, the subnanosecond fluorescence from tryptophan in the same protein incorporated in pure DOPC and mixed DOPC/DOPG as well as mixed DMPC/DMPG lipids with various L/P ratios at different temperatures has been investigated in more detail. A model will be presented here, from which not only the anomalous time-dependent fluorescence anisotropy is interpreted but also information about whole-protein and segmental motion is obtained. In the experiments, two different cases have been distinguished. At temperatures above the phase transition, the rotational diffusion of the entire protein in DOPC and mixed DOPC/DOPG lipid bilayers, has been detected to provide information about the aggregation state. In the case of mixed DMPC/DMPG lipid bilayers below the phase transition temperature, the motion of the entire protein has been found to be so slow that the subnanosecond anisotropy of tryptophan only reflects the segmental motion.

Theory

The fluorescence anisotropy of a tryptophan residue in a protein, incorporated into phospholipid bilayers, arises from the motion of the whole protein and from segmental motion. We denote the anisotropy resulting from the motion of the whole protein and of the protein segment as $r_w(t)$ and $r_s(t)$, respectively. When these motions are independent of each other, the total anisotropy, $r(t)$, of the tryptophan is their product: $r_w(t)\, r_s(t)$ (Jähnig 1979; Kawato and Kinosita 1981).

The whole protein motion may be a restricted motion within a cone around the normal to the membrane (Vaz et al. 1979) and/or a rotational diffusion around this normal (Kawato and Kinosita 1981). An approximate theory that can be used to analyse this motion has been presented by Van der Meer et al. (1984). Since the tryptophan residue in M13 coat protein is located in the hydrophobic domain, it must be positioned within the bilayer (Nozaki et al. 1976) and the head groups of the lipid will severely restrict the wobbling motion of this domain. Therefore, its contribution to the fluorescence anisotropy in the nanosecond regime can be thought to be time-independent. To simplify calculations, we further assume that the protein is rod-shaped and that the emission moment of the fluorophore makes an angle θ with the membrane normal. In this case, the anisotropy decay induced by the rotational diffusion of the whole protein around the membrane normal can be approximated by (Kawato and Kinosita 1981):

$$r_w(t)/r_w(0) = 3\sin^2\theta\cos^2\theta\exp(-t/\phi_r) + \tfrac{3}{4}\sin^4\theta\exp(-4t/\phi_r) + \tfrac{1}{4}(3\cos^2\theta - 1)^2 . \quad (1)$$

With vanishing angle θ, no anisotropy will be detected, as may be expected. The rotational correlation time characteristic for diffusion of the protein about the membrane normal, ϕ_r, can be roughly estimated from the temperature (T), the membrane viscosity (η), the protein shape factor (F) and the effective volume (V) by the Stokes-Einstein relation:

$$\phi_r = 6\eta F V/kT , \quad (2)$$

where k is the Boltzmann constant. This equation indicates that the aggregation state of the protein can be estimated from the measurement of this correlation time (Cherry et al. 1978; Zidovetzki et al. 1981).

The initial value (at $t=0$) of the right side of (1) equals unity. At short times, the slope with respect to time of (1) can be approximated by $-3\sin^2\theta/\phi_r$, at $t=0$. The rotational correlation time of bacteriorhodopsin in DMPC bilayers is about 10 ms at 5 °C above the phase transition (Cherry and Godfrey 1981). This indicates that this rotation, in general, is very slow, as compared to the segmental and local motion occurring in the (sub)nanosecond regime (Datema et al. 1987a). Using this information, (1) can be approximated in the subnanosecond regime by a single exponential function in such a way that the initial value as well as the derivative with respect to time of this equivalent function are equal to those calculated from (1) (Lipari and Szabo 1980). The total anisotropy can then be written as

$$r(t) = r_w(0)\, r_s(t)\exp(-t/\phi_e) , \quad (3)$$

where the equivalent rotational correlation time, ϕ_e, equals $\phi_r/3\sin^2\theta$.

Within the protein subunits, the tryptophan residue, as a segment, may undergo a restricted motion (Lakowicz et al. 1983). If the tryptophan is situated in a homogeneous system this motion can then be described by a correlation time ϕ_s and a constant residual anisotropy $r_s(\infty)$, provided that the protein is also rod-shaped (Kinosita et al. 1977):

$$r_s(t) = \beta_s \exp(-t/\phi_s) + r_s(\infty), \quad (4)$$

where β_s is the difference between the initial and the residual anisotropy: $\beta_s = r_s(0) - r_s(\infty)$.

Substitution of (4) into (3) leads to

$$r(t) = r_w(0)\,\beta_s\{\exp(-t/\phi_s) + S^2/(1-S^2)\}\exp(-t/\phi_e), \quad (5)$$

where the fact that ϕ_e is much longer than ϕ_s has been taken into account. In (5), the order parameter of the transition moment of the tryptophan residue, S, is $\sqrt{r_s(\infty)/r_s(0)}$ (Heyn 1979).

Because the tryptophan residue, in general, is located in different environments, the time-dependent anisotropy resulting from the motion of the tryptophan residue will show an anomalous behaviour (Hudson et al. 1986; Szmacinski et al. 1987; Ludescher et al. 1987), which is very similar to the (sub)nanosecond fluorescence anisotropy of extrinsic probes, such as parinaric acid (Wolber and Hudson 1982) or octadecyl rhodamine B (see preceding paper) in the same M13-coat protein-phospholipid system. As a simplification, we assume that there exist only two distinct sites for the tryptophan residue: site 1 (denoted by subscript 1) and site 2 (denoted by subscript 2), where not only the local anisotropies, but also the local lifetimes may be different. As long as the difference in the two lifetimes is small, the total anisotropy of the tryptophan residue in this protein-lipid system can be approximated as the sum of contributions from the local restricted motion in the two sites and one resulting from the difference in the local lifetimes, τ_{s1} and τ_{s2} (see preceding paper):

$$r_s(t) = (1-C)\,\beta_{s1}\exp(-t/\phi_{s1}) + C\,\beta_{s2}\exp(-t/\phi_{s2}) + C\{r_{s2}(\infty) - r_{s1}(\infty)\}\exp\{t(1/\tau_{s1} - 1/\tau_{s2})\} + r_{s1}(\infty), \quad (6)$$

where C is the relative fluorescence contribution of the tryptophan in site 1 as compared to that of site 2.

Materials and methods

DOPC, DOPG, DMPC and DMPG were purchased from Sigma Chemical Co., St. Louis. All lipids were of 98–99% purity and were used without further purification. *E. coli* K37 and M13 bacteriophage were a gift from Dr. B. Harmsen, University of Nijmegen. Using purified bacteriophage, phenol-extracted M13 coat protein was prepared according to the procedure of Knippers and Hoffmann-Berling (1966). The coat protein, then, was incorporated in phospholipids with various lipid to protein ratios (L/P), as described previously (Datema et al. 1987a, b).

The time-resolved single photon counting apparatus in our laboratory has been described previously (Visser and Van Hoek 1979; Van Hoek et al. 1983). The excitation wavelength in the present experiments is 300 nm. The fluorescence of the tryptophan is detected through an interference filter at 339 nm (Schott, band width 5 nm). As compared to the previous study (Datema et al. 1987a), the time-resolution of the detection system has been improved by incorporation of a microchannel plate detector (Hamamatsu R 1645 U-01) (Visser et al. 1988).

The total fluorescence is the sum of the measured polarized components parallel, $I_{\parallel}$, and perpendicular, $I_{\perp}$, to the electric vector of the excitation:

$$S(t) = I_{\parallel}(t) + 2I_{\perp}(t), \quad (7)$$

where the background fluorescence from lipids only in the two directions must be subtracted from the corresponding components, taking into account the proper weighting scheme (Vos et al. 1987). The sum is the convolution of the real (deconvoluted) fluorescence decay $s(t)$. The real decay is expanded into a sum of exponential functions:

$$s(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad (8)$$

in which α_i is the fractional contribution of the ith component with lifetime τ_i. The parameters, α_i and τ_i can be determined from $S(t)$ by the least-squares method, in which the weighted sum of the squared residuals between the measured decay and fitted decay is minimized. To determine the system impulse response $P(t)$, a reference compound with a single exponential decay of lifetime τ_r, is measured. Based upon the calculation of the Laplace transform and its inverse transform, (7) is also related to the reference measurement (Löfroth 1985; Vos et al. 1987). In the present experiments p-terphenyl in ethanol has been used as the reference compound, of which the lifetime is 1.06 ns (Vos et al. 1987).

The average lifetime is defined as

$$\langle\tau\rangle = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i . \quad (9)$$

The anisotropy is defined as the ratio of the difference between the two deconvoluted polarized components to their sum. Despite the possible non-exponential property of the anisotropy, we assume that the anisotropy can be expanded into a series of exponentials:

$$r(t) = \sum_j \beta_j \exp(-t/\phi_j), \quad (10)$$

where ϕ_j is the correlation time, its fractional contribution is related to β_j. The measured polarized components can be expressed as the convolution of a known reference compound and the functions, related to the anisotropy and the known real fluorescence decay too. With β_j and ϕ_j one can fit the two polarized components simultaneously in a globular approach (Cross and Fleming 1984; Vos et al. 1987).

The quality of fit is judged by the weighted residuals, the autocorrelation function of those residuals, the reduced chi square (χ^2) value, the Durbin-Watson parameter and the number of zero passages of the autocorrelation functions (O'Connor and Phillips 1984).

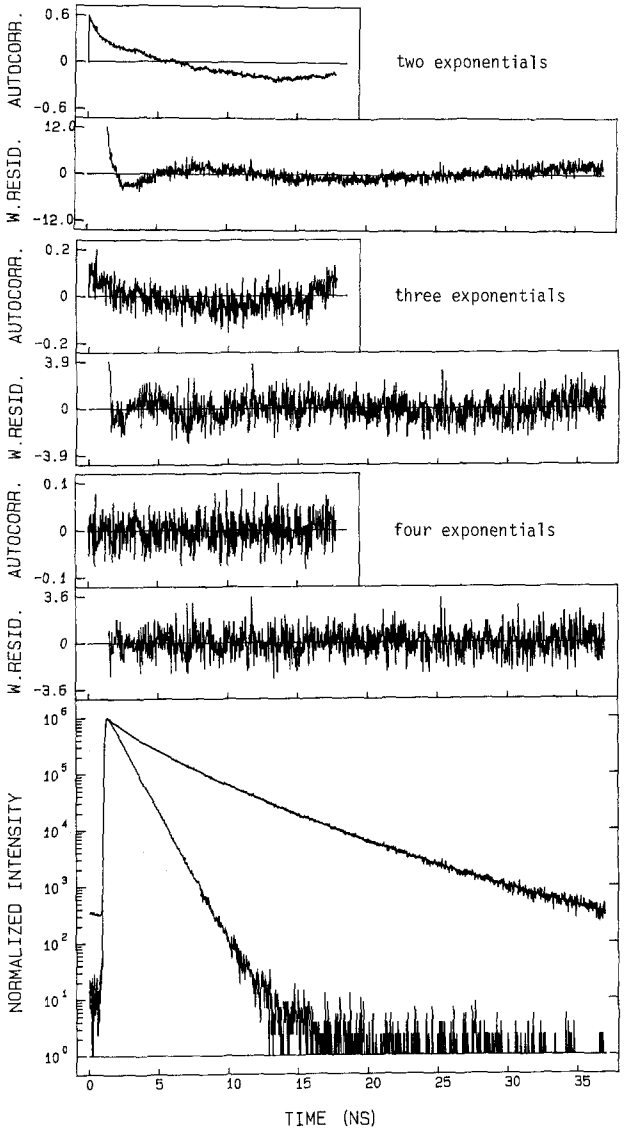

Fig. 1. Fluorescence decay at 339 nm of the single tryptophan residue in bacteriophage M13 coat protein incorporated in DMPC/DMPG (80/20, w/w) after excitation at 300 nm at 0 °C. Also shown is the fluorescence response of *p*-terphenyl in ethanol. The L/P ratio is 8. The fit channel range is from 40 to 1024 (36 ps/channel). The parameters describing the fit with 4 exponentials are $\alpha_1 = 0.18 \pm 0.01$, $\alpha_2 = 0.33 \pm 0.02$, $\alpha_3 = 0.46 \pm 0.02$, $\alpha_4 = 0.04 \pm 0.01$, $\tau_1 = 0.37 \pm 0.05$ ns, $\tau_2 = 1.89 \pm 0.11$ ns, $\tau_3 = 4.65 \pm 0.16$ ns, $\tau_4 = 10.25 \pm 0.72$ ns. The upper panels represent the residuals and the autocorrelation function of the residuals for the fit with 2, 3 and 4 exponentials, respectively. The χ^2 values for these fits are 2.30, 1.12 and 0.99, respectively. The Durbin-Watson parameters are 0.68, 1.76 and 1.97, respectively. The numbers of zero passages in the autocorrelation function are 14, 188 and 246, respectively

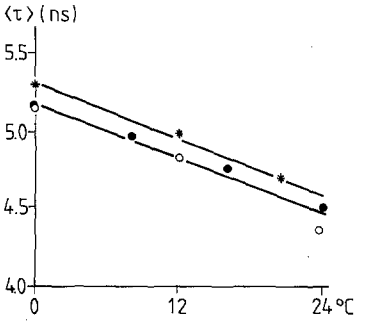

Fig. 2. Average fluorescence lifetime at 339 nm as a function of temperature (°C) of the single tryptophan residue of bacteriophage M13 coat protein incorporated in DOPC (–o–), DOPC/DOPG (80/20 w/w) (–●–) and mixed DMPC/DMPG (80/20 w/w) (–*–) with L/P = 35, 41, 38, respectively, after excitation at 300 nm

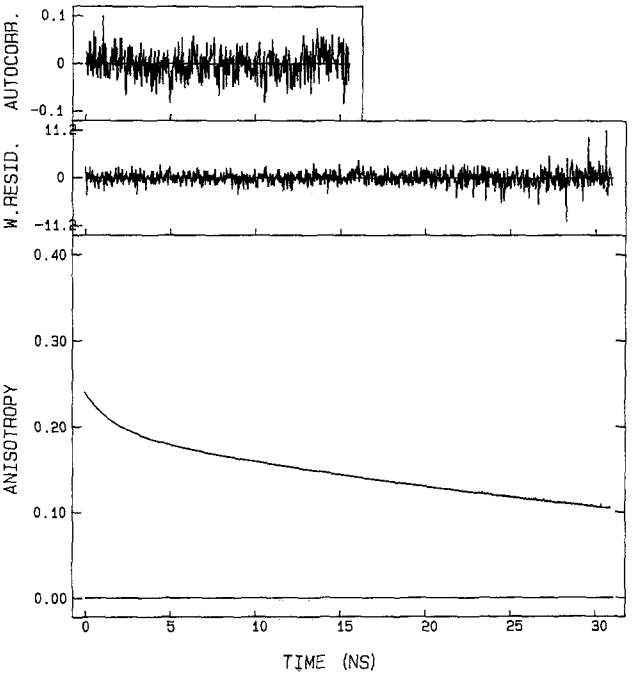

Fig. 3. Fluorescence anisotropy decay (339 nm) at 24 °C of the single tryptophan residue in bacteriophage M13 coat protein incorporated in DOPC bilayers with L/P = 35 after excitation at 300 nm. The fit from channels 40 to 1024 (33 ps/channel) is a dual exponential function with $\beta_1 = 0.056 \pm 0.003$, $\beta_2 = 0.196 \pm 0.003$, $\phi_1 = 1.6 \pm 0.2$ ns and $\phi_2 = 50 \pm 4$ ns. The χ^2 value and the Durbin-Watson parameter are 0.98 and 1.96, respectively. The number of zero passages of the parallel intensity component is 230, of the perpendicular intensity component is 264

Results

In general, the fluorescence decay of even single tryptophan-containing proteins has been found to be multi-exponential (Munro et al. 1979). This is also found for the tryptophan in M13 coat protein incorporated in lipid bilayers, as shown in Fig. 1. A comparison of the best fits of the fluorescence decays for the tryptophan into a dual, triple, and quadruple exponential function shows the highest fit quality for four exponential functions, in agreement with other work (Johnson and Hudson 1989). Therefore, this expansion has been used to fit the fluorescence decay of tryptophan in all lipid-protein systems.

The fluorescence lifetimes and the corresponding pre-exponential coefficients of tryptophan, measured at 24 °C, for the M13 coat protein, incorporated in either DOPC or a mixture of DOPC and DOPG (80/20 w/w)) with different L/P ratios, are given in Table 1. The same table

Table 1. Fluorescence decay parameters of the tryptophan residue in bacteriophage M13 coat protein in various DOPC/DOPG and DMPC/DMPG lipid systems. The χ^2 value and the Durbin-Watson parameter are about 1.0 and 2.0, respectively. The lifetimes τ_1 and τ_4 were fixed in the analysis

Lipid	Temp °C	L/P	α_1 ±0.02	τ_1 ns	α_2 ±0.02	τ_2 ns ±0.1	α_3 ±0.02	τ_3 ns ±0.1	α_4 ±0.003	τ_4 ns	$\langle\tau\rangle$ ns
DOPC	24	8	0.29	0.47	0.35	1.7	0.39	4.1	0.014	10.1	3.6
		35	0.15	0.47	0.32	1.8	0.51	4.6	0.024	10.1	4.4
		72	0.07	0.47	0.33	2.1	0.59	5.0	0.017	10.1	4.7
DOPC/DOPG	24	8	0.17	0.46	0.35	2.02	0.46	4.58	0.022	11.1	4.4
		41	0.15	0.46	0.33	2.02	0.50	4.58	0.027	11.1	4.6
		62	0.14	0.46	0.32	2.02	0.51	4.58	0.028	11.1	4.6
DMPC/DMPG	20	8	0.16	0.50	0.35	1.8	0.46	4.3	0.035	9.8	4.3
		17	0.14	0.50	0.35	1.9	0.48	4.5	0.037	9.8	4.5
		38	0.13	0.50	0.36	2.1	0.48	4.7	0.037	9.8	4.6
		83	0.12	0.50	0.35	2.1	0.49	4.8	0.038	9.8	4.7
		102	0.11	0.50	0.37	2.2	0.49	5.0	0.030	9.8	4.7

Table 2. Anisotropy decay parameters of the tryptophan residue at 24 °C in bacteriophage M13 coat protein in various DOPC/DOPG lipid systems. The χ^2 value and the Durbin-Watson parameter are about 1.0 and 2.0, respectively

Lipid	L/P	β_s ±0.005	ϕ_s ns ±0.2	$r_s(\infty)$ ±0.003	ϕ_e ns ±6.0
DOPC	8	0.044	0.8	0.225	66.0
	35	0.056	1.6	0.196	50.0
DOPC/DOPG	8	0.053	1.4	0.198	70.0
	41	0.051	1.0	0.206	62.0
	62	0.050	1.1	0.204	58.0

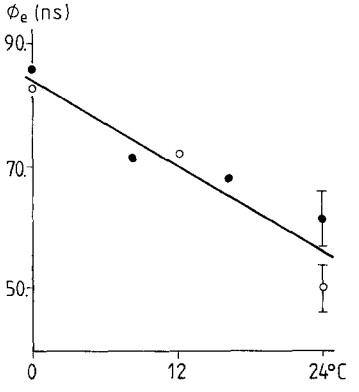

Fig. 4. Equivalent rotational correlation time (339 nm) of the single tryptophan residue in the bacteriophage M13 coat protein incorporated in DOPC (–o–) and mixed DOPC/DOPG bilayers (80/20 w/w) (–●–) as a function of temperature (°C) after excitation at 300 nm. The L/P ratio is 35 and 41, respectively

shows the decay parameters at 20 °C for the M13 coat protein-DMPC/DMPG (80/20 w/w) lipid system. The average lifetime as a function of temperature is shown in Fig. 2. The average lifetime decreases with decreasing L/P ratio and increasing temperature.

Since the experiments for the different DOPC/DOPG lipid systems were performed at about 22–46 °C above the phase transition temperature (−22 °C, for DOPC lipid, see Chapman et al. 1967), the effect of the protein rotation on the fluorescence anisotropy of the tryptophan in this lipid-protein system may be significant. Assuming, for simplicity, a uniform environment for the tryptophan, the anisotropy may then be fitted to a dual exponential function, see (5). Figure 3 shows the anisotropy decay at 24 °C, corresponding to the DOPC lipid with L/P ratio equal to 35. The anisotropy decay parameters at 24 °C for the DOPC as well as the mixed DOPC/DOPG lipid (80/20 w/w) system with different L/P ratios are summarized in Table 2. For the protein-DOPC and DOPC/DOPG lipid system with L/P ratio equal to 35 and 42, respectively, the equivalent rotational correlation time as function of temperature is presented in Fig. 4.

The anisotropy decay for the M13 coat protein-DMPC/DMPG (80/20 w/w) lipid system was measured at 20 °C, which is just below its phase transition temperature (23 °C, see Datema et al. 1987b). Using the fact that the rotational correlation time of bacteriorhodopsin in the DMPC lipid equals 3.5–16 ms at the same temperature (Cherry and Godfrey 1981), the rotational correlation time will be so long that in the nanosecond regime its corresponding contribution to the total anisotropy can be considered to be time-independent. The total anisotropy then reflects only the segmental motion of the tryptophan residue in the protein matrix. Considering heterogeneity of the tryptophan environment, the anisotropy decay is fitted to a triple exponential function with a constant value, see (6). Figure 5 shows the fit of the anisotropy decay at 20 °C with L/P ratio equal to 8, which contains a contribution with a negative pre-exponential coefficient and correlation time. The anisotropy parameters for the experiments, performed at 20 °C, are listed in Table 3. To avoid a possible correlation between the fitting parameters, ϕ_2 was fixed to the value obtained at L/P 102 (which approaches the value for a pure lipid system) for experiments performed at the same temperature.

Table 3. Anisotropy decay parameters of the tryptophan residue in bacteriophage M13 coat protein in mixed DMPC/DMPG (80/20 w/w) lipid systems. The χ^2 value and the Durbin-Watson parameter are about 1.0 and 2.0, respectively. In the upper table ϕ_2 was fixed in the analysis

Temperature 20 °C							
L/P	β_1 ±0.008	ϕ_1 ns	β_{s2} ±0.02	ϕ_2 ns	β_3 ±0.07	ϕ_3 ns	β_4
8	0.053	2.3(±0.5)	0.03	0.3	−0.02	−20.0 (±8.0)	0.20(±0.02)
17	0.052	3.0(±0.6)	0.04	0.3	−0.03	−30.0 (±30.0)	0.21(±0.04)
38	0.055	3.0(±0.6)	0.04	0.3	−0.02	−20.0 (±10.0)	0.20(±0.02)
83	0.054	4.0(±0.7)	0.04	0.3	−0.02	−30.0 (±40.0)	0.20(±0.05)
102	0.050	4.5(±1.0)	0.07	0.3	−0.03	−100.0(±150.0)	0.20(±0.07)
L/P = 8							
Temp °C	β_1 ±0.008	ϕ_1 ns	β_{s2} ±0.02	ϕ_2 ns	β_3 ±0.07	ϕ_3 ns	β_4
0	0.049	5.0(±2.0)	0.03	0.7(±0.3)	−0.02	−30.0 (±30.0)	0.21(±0.04)
12	0.055	4.0(±1.0)	0.03	0.5(±0.2)	−0.02	−30.0 (±30.0)	0.20(±0.03)
20	0.053	2.3(±0.5)	0.03	0.3(±0.2)	−0.02	−20.0 (±8.0)	0.20(±0.02)
30	0.052	2.3(±0.4)	0.04	0.3(±0.2)	−0.02	−20.0 (±6.0)	0.20(±0.02)

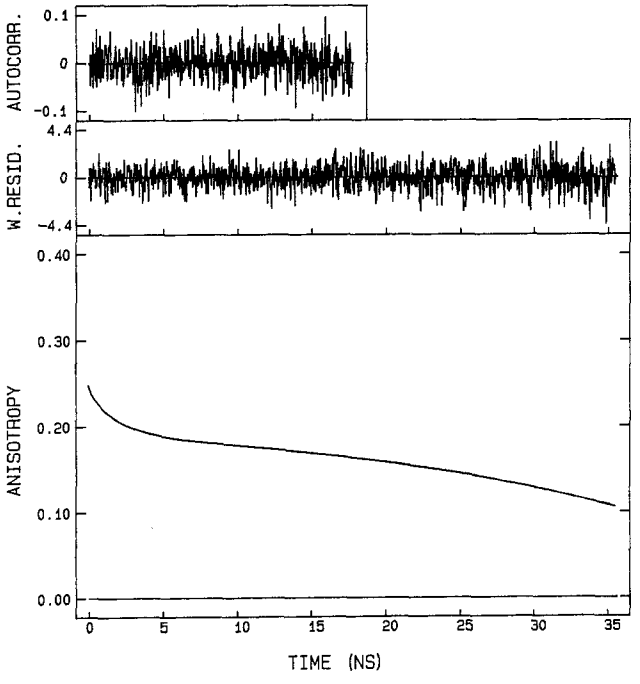

Fig. 5. Fluorescence anisotropy decay (339 nm) of the single tryptophan residue in bacteriophage M13 coat protein incorporated in DMPC/DMPG (80/20 w/w) with L/P = 8 after excitation at 300 nm at 20 °C. The fit from channels 40 to 1024 (36 ps/channel) is a triple exponential function with a constant. The anisotropy parameters are $\beta_1 = 0.027 \pm 0.016$, $\beta_2 = 0.053 \pm 0.005$, $\beta_3 = -0.017 \pm 0.014$, $\beta_4 = 0.204 \pm 0.018$, $\phi_1 = 0.34 \pm 0.2$ ns, $\phi_2 = 2.31 \pm 0.47$ ns, $\phi_3 = -20.0 \pm 8.0$ ns. The χ^2 value and the Durbin-Watson parameter are 0.94 and 2.07, respectively. The number of zero passages of the parallel intensity component is 239 and of the perpendicular intensity component, 288

Discussion

Fluorescence decay

After UV-excitation the indole moiety of tryptophan can be excited into two energy levels, ^1L_a and ^1L_b, of which the spectral distributions overlap within 250–300 nm (Andrews and Forster 1974; Valeur and Weber 1977).The best fit to the fluorescence decay of tryptophan, therefore, is at least a dual exponential function arising from dual emission. The theory describing this process (rapid internal conversion between ^1L_a and ^1L_b) has been presented by Cross et al. (1983). The fluorescence decay of indole is very sensitive to the photophysics of indole and to its surroundings. Small changes in the conformation, for instance, may lead to a large change in radiationless decay of the indole (Creed 1984). Due to microheterogeneity, the fluorescence decay in the lipid-protein system, such as M13 coat protein incorporated in lipids, is expected to display an even more complex pattern and has to be fitted with more lifetime components.

As shown in Table 1, the fluorescence of the tryptophan in the M13 coat protein incorporated in phospholipids can be best described by a combination of four lifetimes of about 0.5, 2.0, 4.5 and 10.0 ns, respectively. The first three lifetimes are similar to those measured by Datema et al. (1987a) in the M13 coat protein-DMPC/DMPG lipid bilayers (about 0.2, 2.2 and 5.5 ns). As mentioned before, the tryptophan located in the hydrophobic domain of the M13 coat protein is isolated by the bilayer from the residues in the N- and C-terminus. The dominant influence on tryptophan fluorescence must then result from interaction between the excited indole and possible quenchers, such as neighbouring amino acid residues (Lumry and Hershberger 1978; Petrich et al.

1983), located in the hydrophobic domain. This may be the reason why the four lifetimes describing the basic fluorescence kinetics are almost independent of the lipids used.

Quenching can explain the fact that the average fluorescence lifetime increases at increasing L/P ratio. Because of the short distance, the tryptophan fluorescence in a protein aggregate may be quenched by the residues of neighbouring proteins (Tanaka and Mataga 1982, 1987). Therefore, the average lifetime of tryptophan fluorescence in aggregated protein is shorter than that for less aggregated protein. A typical example is the fluorescence decay of the tryptophan in the coat protein in the mixed DOPC/DOPG (80/20 w/w) lipid bilayers. The lifetimes are 0.46, 2.02, 4.58, and 11.1 ns, respectively, independent of the L/P ratio. The relative contribution of the fast decay times (i.e. the first two) to that of the slow ones decreases from 1.08 to 0.85 as the L/P ratio increases from 8 to 62 (see Table 1). The evidence of the change in lifetime components is the increase of two out of four lifetimes for the tryptophan in the M13 coat protein-pure DOPC lipid system from 1.7 to 2.1 ns and from 4.1 to 5.0 ns, respectively, within the L/P range from 8 to 72.

The plots of the average lifetime of the tryptophan in various lipid bilayers as a function of temperature show a shortening (Fig. 2), which provides further evidence for the well-known behaviour of dynamic quenching of the fluorescence of tryptophan-containing compounds (Chang et al. 1983).

Fluorescence anisotropy

A number of experiments has indicated that the time-resolved anisotropy of fluorescent probes in membranes should tend to a finite non-zero residual anisotropy (Zannoni et al. 1983; Ameloot et al. 1984). However, as seen in Figs. 3 and 5, this tendency is not observed in our time-resolved fluorescence anisotropy experiments, where the protein with tryptophan may be regarded as a fluorescent probe of phospholipid bilayers.

In DOPC and mixed DOPC/DOPG bilayers, two distinct kinds of rotational correlation times are apparent from the anisotropy decay of the tryptophan of the M13 coat protein. The fast correlation time (about 1–2 ns) may be attributed to the motion of the tryptophan residue, while the slow one is related to the motion of the whole protein or aggregate. The equivalent rotational correlation time describing the rotation of the whole protein around the membrane normal decreases with increasing temperature (Fig. 4). Such a behaviour is predicted by (2). As seen in Table 2, at the same temperature (24 °C) the rotational correlation time is a strong function of the L/P ratio and it decreases when this ratio is increasing. In turn, the effective volume of protein (aggregate) will also decrease, as seen from (2). It is evident that in the case of lower L/P ratio, the proteins in the bilayer preferably aggregate to a larger particle.

Using (5), the order parameter of the transition moment of the tryptophan residue in the protein matrix for different DOPC/DOPG lipid systems at different temperatures above the phase transition temperature is calculated to be about 0.9.

The anisotropy decay of the tryptophan residues in the M13 coat protein incorporated in the mixed DMPC/DMPG (80/20, w/w) lipid system demonstrates an unusual behaviour (Fig. 5). The fitted anisotropies at 20 °C, as shown in Table 3, contain a contribution from a fast motion with correlation time of about 0.3 ns. This motion as well as the slower motion show a temperature-dependence. Such behaviour and a negative decay time suggest the location of tryptophan residues in binary sites. From (6), it can be shown that the negative decay time is related to the difference of the fluorescence lifetimes of the tryptophan in two distinct sites, the lifetime of tryptophan in site 1 being shorter than that in site 2. The fact that the fluorescence lifetime of the tryptophan will shorten due to quenching between proteins suggests that the site 1 for the tryptophan in the protein-DMPC/DMPG system should be located within an aggregate, while site 2, corresponding to the fast motion, is linked to the tryptophan exposed to lipid molecules. For this reason ϕ_2 was fixed in the analysis to the value obtained at L/P 102. Under the assumption that the initial anisotropies of the tryptophan in these two sites are the same, and by comparing the pre-exponential coefficients of (6) with those in Table 3, we can estimate the relative fluorescence contribution from the tryptophan within aggregates to that facing lipids to be about 0.7. The order parameters of the transition moment of the tryptophan residue in the two sites are about 0.9 and 0.5, respectively.

Comparing the correlation times and the order parameters of the tryptophan residue of M13 coat protein incorporated within the various DOPC and DOPG bilayers to those in the mixed DMPC/DMPG bilayers, supports the suggestion that the fast correlation time and the order parameter, calculated from the anisotropy in the protein-DOPC/DOPG lipid systems, in fact, describe the motion of the tryptophan residue within aggregate. The faster motion of the tryptophan exposed to lipid may be overlooked in the calculations.

The constancy of the order parameter for different lipid-protein matrices (i.e. L/P ratio, different lipids) and temperatures implies that the tryptophan may be isolated from the lipids by the neighbouring proteins. The wobbling space is expected to be strongly restricted and dependent on the protein microstructure near this residue, and not on lipid matrix. Within the aggregate the semi-angle of the cone, in which the transition moment of the tryptophan wobbles, is estimated to be about 25°, as compared to about 50° for the tryptophan exposed to the lipids (Heyn 1979; Kinosita et al. 1982).

In our analysis, the fluorescence decay has been decomposed into four lifetime components, and the segmental motion has been considered as binary. Here, the concept of multiple environments for the tryptophan residue has been tremendously simplified. Because of the continuity of microenvironment of the indole moiety, not only the lifetimes but also the motions of the tryptophan residues should be considered to be distributed continuously as function of their corresponding fractional contributions (Alcala et al. 1987a, b). The four discrete lifetimes

components and the two discrete correlation times in the fitted fluorescence decay and anisotropy decay in the present experiments may be the approximate average values in their corresponding range (Ludescher et al. 1985). For precise description of dynamics of protein in heterogeneous environments, distributions of lifetimes and correlation times must be taken into consideration.

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