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

I. Application to the lipid probe octadecyl rhodamine B in interaction with bacteriophage M13 coat protein incorporated in phospholipid bilayers

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Abstract. Fluorescent probes located in heterogeneous environments give rise to anomalous time-resolved fluorescence anisotropy. A simple analytical expression of anisotropy has been derived for the case of a small difference in local fluorescence lifetimes. The expression has the diagnostic advantage that the time dependence of the fluorescence anisotropy can be predicted from the differences in fluorescence lifetimes and residual anisotropies of the probes located in different sites. Using this model, the local fluorescence anisotropy parameters and the relative contributions of the lipid probe octadecyl rhodamine B in a lipid environment and in the vicinity of bacteriophage M13 coat protein reconstituted in phospholipid bilayers, composed of 80% 1,2-dimyristoyl-sn-glycero-3-phosphocholine and 20% 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol have been determined experimentally. At 40 °C, the correlation times for bound and free probes are 2.3 and 3.0 ns, respectively, while the corresponding order parameters are 0.85 and 0.62, respectively.

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

Introduction

Time-resolved fluorescence depolarization measurements have been used to reveal the molecular dynamics of various biological systems (Van Hoek et al. 1987). By means of this technique as well as with ESR spin label experiments, the interaction between the bacteriophage M13 coat protein and various synthetic lipid bilayers has been studied to understand the mechanism of virus infection (Datema et al. 1987a, b).

The time-resolved anisotropy of fluorescent probes in pure lipid bilayers has been well studied. It may be described by a multiple exponential function and a finite constant (residual anisotropy) on a suitable time-scale of the fluorescent experiment (Kinosita et al. 1977; Zannoni et al. 1983; Lipari and Szabo 1980; Van der Meer et al. 1984). However, this tendency in the nanosecond time regime may not be observed for lipid-protein systems, and the time-dependent anisotropy may then appear to be non-exponential, showing an initial decay followed by a rise (Wolber and Hudson 1982; Van Paridon et al. 1988). This phenomenon has been interpreted as the result of microheterogeneity of environments for the probes (Ludescher et al. 1987). The photophysical behavior of a fluorescent membrane probe may be very sensitive to the influence of a protein, and varies as a function of distance between them. This may then result into a variation of local lifetime and/or local fluorescence anisotropy of probes and, consequently, the fluorescence anisotropy for this system shows an unusual behavior.

It is of interest not only to interpret the fluorescence anisotropy of probes incorporated in microheterogeneous biosystems, but also to extract information on the local probe motion. This paper is devoted to an approach to solve this problem. We have applied this approach to the analysis of the anisotropy decay of octadecyl rhodamine B chloride, as a fluorescent lipid probe, in a system of bacteriophage M13 coat protein, incorporated in phospholipid bilayers, composed of 80% 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 20% 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG). This amphiphilic probe has been used previously in studies of membrane fusion (Hoekstra et al. 1984), and dynamics of micelles (Ediger et al. 1984; Visser et al. 1988). In the accompanying paper we use a similar approach to describe the fluorescence anisotropy of the single tryptophan in the bacteriophage M13 coat protein.

Abbreviations: ESR, electron spin resonance; 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

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Theory

To simplify calculations, the multiple environments for fluorophores may be restricted to two sites only: one part of probe molecules, called bound probes and denoted by a subscript b of "bound", is located in the neighbourhood of the protein and another part of probe molecules (denoted by f of "free") is located in the bulk lipids remote from protein. It is assumed that there is not exchange between the two sites on a nanosecond time scale. When the fluorescence decay in each environment is mono-exponential, each fluorescence intensity, $I_j(t)$, can be written as

$$I_j(t)=C_j\exp(-t/\tau_j), \quad j=b \quad \text{or} \quad f, \tag{1}$$

where τ_j is the lifetime and C_j is the initial intensity. The ratio, C_b/C_f, will be called the relative fluorescence contribution C. When the local anisotropies corresponding to each environment, $r_j(t)$, are not correlated to each other, the apparent anisotropy of the total system is given by (Lakowicz 1983)

$$r(t)=\frac{r_f(t)\exp(-t/\tau_f)+C\,r_b(t)\exp(-t/\tau_b)}{\exp(-t/\tau_f)+C\exp(-t/\tau_b)}. \tag{2}$$

The difference, $\Delta\tau$, between the lifetimes τ_b and τ_f is now assumed to be small, as compared with the lifetime itself. Then we have the approximation

$$1/\tau_b=1/(\tau_f+\Delta\tau)\cong 1/\tau_f-\Delta\tau/\tau_f^2. \tag{3}$$

Substituting (3) into (2), the anisotropy is obtained, expressed in terms of $\Delta\tau/\tau_f^2$. As long as time is not very long (i.e. $t\ll|\tau_f^2/\Delta\tau|$), (2) can be approximated as

$$r(t)=\{1/(1+C)^2\}\,[(1+2C)\,r_f(t)+C\{(1+2C)\,r_b(t)-r_f(t)\}\exp(t\,\Delta\tau/\tau_f^2)-C^2 r_b(t)\exp(2t\,\Delta\tau/\tau_f^2)]. \tag{4}$$

With vanishing lifetime difference ($\Delta\tau=0$), (4) is simply the sum of two C-dependent contributions arising from motions of the fluorophore in the different environments.

Equation (4) can be further simplified if the fluorescence contribution from bound probes is small (i.e. $C\ll 1$):

$$r(t)=r_f(t)+C\{r_b(t)-r_f(t)\}\exp(t\,\Delta\tau/\tau_f^2), \tag{5}$$

where the terms containing C of order higher than 2 are neglected.

For fluorophores situated in an ordered environment (i.e. a lipid membrane), the anisotropy can be approximately described by a restricted diffusional motion with correlation time ϕ_j and a residual anisotropy $r_j(\infty)$, which is related to the probe orientational order (Kinosita et al. 1977):

$$r_j(t)=\beta_j\exp(-t/\phi_j)+r_j(\infty), \tag{6}$$

where β_j is the difference between the initial and the residual anisotropy, i.e. $\beta_j=r_j(0)-r_j(\infty)$.

Substitution of (6) into (4) leads to an expression of anisotropy. This expression contains two exponential terms with decay parameter $1/(1/\phi_j+\Delta\tau/\tau_j^2)$, which can be simply replaced by ϕ_j, because $\Delta\tau/\tau_j^2$ has been assumed to be small. Therefore, we have

$$r(t)=\{1/(1+C)\}\,\beta_f\exp(-t/\phi_f)+\{C/(1+C)\}\,\beta_b\exp(-t/\phi_b)+\{C/(1+C)^2\}\cdot[\{(1+2C)\,r_b(\infty)-r_f(\infty)\}\exp(t\,\Delta\tau/\tau_f^2)-C\,r_b(\infty)\exp(2t\,\Delta\tau/\tau_f^2)]+[1-\{C/(1+C)\}^2]\,r_f(\infty). \tag{7}$$

In the case of a relatively low fluorescence contribution from bound probes ($C\ll 1$), (5) and (6) result in

$$r(t)=(1-C)\,\beta_f\exp(-t/\phi_f)+C\,\beta_b\exp(-t/\phi_b)+C\,\{r_b(\infty)-r_f(\infty)\}\exp(t\,\Delta\tau/\tau_f^2)+r_f(\infty). \tag{8}$$

There are two interesting cases worth noting, in which the apparent anisotropy of the system may increase with time. When the residual anisotropy of the bound probes is larger than that of the free probes, the positive lifetime difference will cause the anisotropy to increase with time, see (8). In the case of negative difference in residual anisotropies of bound and free probes, a negative lifetime difference also results in an increase of anisotropy. When most fluorescence is contributed by protein-bound probes ($C\gg 1$), one may directly estimate the residual anisotropy for the bound probe by interchanging the subscripts in (8).

We further assume that the influence of the protein on the properties of the free probe is negligible in such a way that the anisotropy parameters describing free probes in (8) can be replaced by those found by a single exponential analysis of the anisotropy decay of the probe in a pure lipid system, see (6). The other parameters in (8) can then be determined by analyzing the fluorescence anisotropy decay data using a triple-exponential function with a known constant anisotropy. This yields information about the characteristics of the bound fluorophores.

In most biological systems the fluorescence decay of incorporated fluorophores turns out to be complex. Several factors may be responsible, for instance microheterogeneity of the probe environment or the involvement of an excited-state reaction. The fluorescence decay is then mostly expanded into a sum of exponential functions or described as a distribution of lifetimes. In case of a series of k exponential terms for the fluorescence decay of free and bound probes, the following generalization of (2) should be considered:

$$r(t)=\frac{r_f(t)\sum_k \alpha_{f,k}\exp(-t/\tau_{f,k})+C\,r_b(t)\sum_k \alpha_{b,k}\exp(-t/\tau_{b,k})}{\sum_k \alpha_{f,k}\exp(-t/\tau_{f,k})+C\sum_k \alpha_{b,k}\exp(-t/\tau_{b,k})}. \tag{9}$$

Using the approximation that $C\ll 1$, it can be calculated that (5) is changed into a more general Eq.

$$r(t)=r_f(t)+C\{r_b(t)-r_f(t)\}\,R, \tag{10}$$

where the factor R is equal to

$$R=\frac{\sum_k \alpha_{b,k}\exp(-t/\tau_{b,k})}{\sum_k \alpha_{f,k}\exp(-t/\tau_{f,k})}. \tag{11}$$

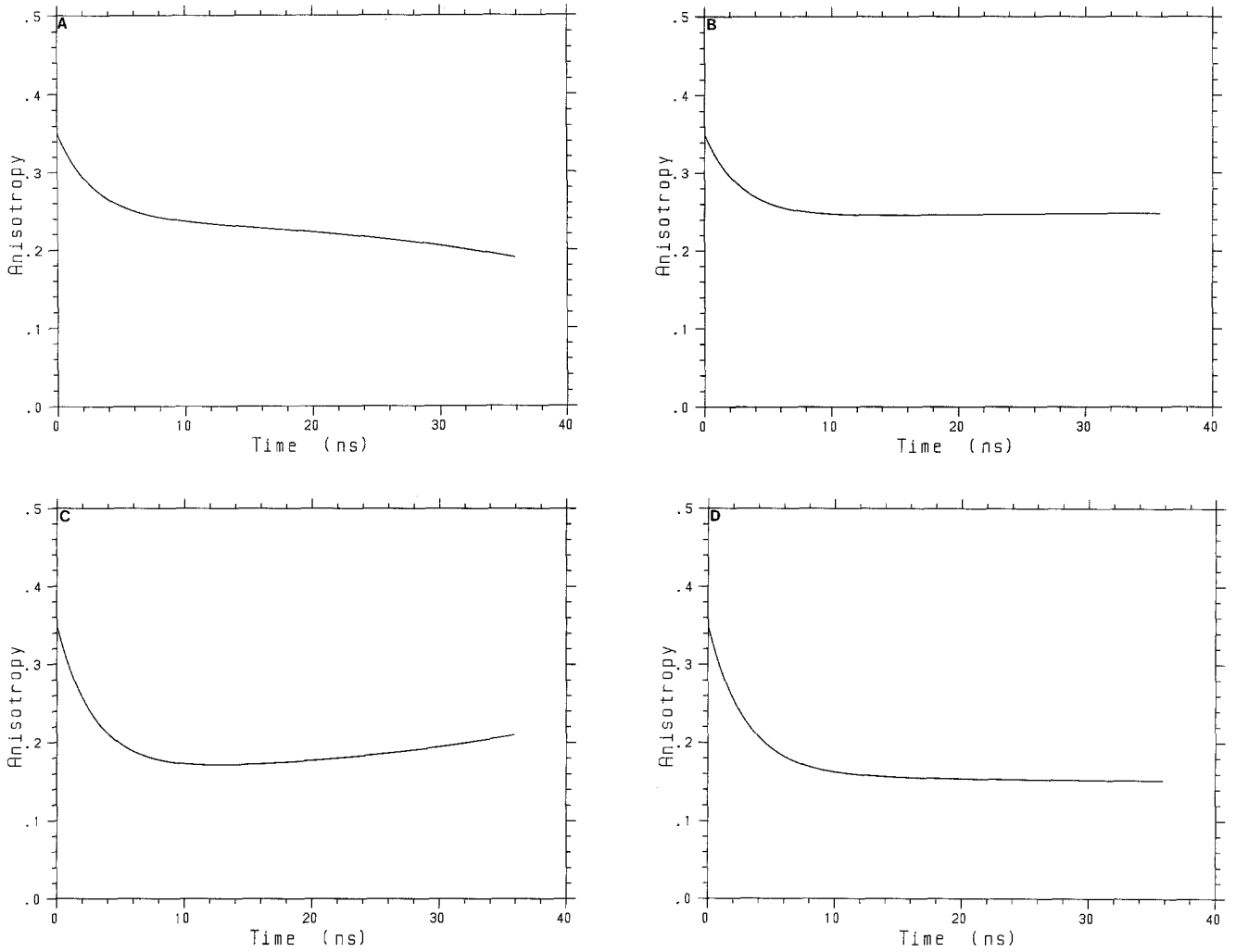

Fig. 1 A–D. Simulations of the time dependence of the fluorescence anisotropy as described by (8), using the following parameters: $C=0.1$, $\phi_f=3.0$ ns, $\phi_b=2.3$ ns and $r(0)=0.35$. The simulations are carried out for four different combinations of $\Delta\tau/\tau_f^2$ and $\Delta r(\infty)=r_b(\infty)-r_f(\infty)$

A $\Delta\tau/\tau_f^2=$ 0.05 ns^{-1}, $\Delta r(\infty)=-0.10$ ($r_b(\infty)=0.15$, $r_f(\infty)=0.25$);
B $\Delta\tau/\tau_f^2=-0.05$ ns^{-1}, $\Delta r(\infty)=-0.10$ ($r_b(\infty)=0.15$, $r_f(\infty)=0.25$);
C $\Delta\tau/\tau_f^2=$ 0.05 ns^{-1}, $\Delta r(\infty)=$ 0.10 ($r_b(\infty)=0.25$, $r_f(\infty)=0.15$);
D $\Delta\tau/\tau_f^2=-0.05$ ns^{-1}, $\Delta r(\infty)=$ 0.10 ($r_b(\infty)=0.25$, $r_f(\infty)=0.15$).
See text for further details

The two multi-exponential functions in (11) can be simplified by considering the second order moments of the fluorescence decay (Lipari and Szabo 1980). By defining an average lifetime $\langle\tau\rangle$ given by

$$\langle\tau\rangle=\sum_k \alpha_k \tau_k^2 / \sum_k \alpha_k \tau_k \tag{12}$$

for each site, the factor R can be shown to become

$$R=\exp(t\,\Delta\tau/\langle\tau_f\rangle^2), \tag{13}$$

for small values of $\Delta\tau=\langle\tau_b\rangle-\langle\tau_f\rangle$, where $\langle\tau_b\rangle$ and $\langle\tau_f\rangle$ are the average lifetimes of the bound and free fluorophores, respectively. Note that (10) with R from (3) is identical to (5) with τ_f replaced by $\langle\tau_f\rangle$. The same modification applies to (8), which will therefore be used in our further analysis.

As stated above, the relatively simple expression of (8) has an important diagnostic advantage. One can predict whether the time-dependent fluorescence anisotropy exhibits a rise after initial decay or whether there is an accelerated decay at longer times. To stress this point, we have carried out some simulations of (8) that are presented in the next section.

Simulations

The simulations (Fig. 1) of the model described by (8) were carried out with the following common parameters that are representive for a fluorescent probe in a membrane: $C=0.1$, $\phi_f=3.0$ ns, $\phi_b=2.3$ ns. The initial anisotropy $r(0)$ is 0.35 for both the free and bound probe. The absolute value of the term $\Delta\tau/\tau_f^2$ is 0.05 ns^{-1} and in the simulations this term is assumed to be positive or negative, depending upon the sign of the lifetime difference $\Delta\tau$. For a probe with $\tau_f=2.0$ ns the corresponding lifetime difference is then 0.2 ns. The absolute value of $\Delta r(\infty)=r_b(\infty)-r_f(\infty)$ is 0.10 and also for this parameter the sign is varied. The simulations were extended to 36 ns, which is about the time span usually available for most fluorophores. It should be noted that the signs of $\Delta\tau$ and

$\Delta r(\infty)$ in (8) determine the characteristics of the time-dependent behaviour of the fluorescence anisotropy $r(t)$.

The simulations in Fig. 1 B and D with $\Delta\tau/\tau_f^2 = -0.05\ \mathrm{ns}^{-1}$, show a time-dependent anisotropy that levels off to a constant value given by $r_f(\infty)=0.25$ and 0.15, respectively. This situation is also observed in fluorescence anisotropy experiments of fluorescent probes in single sites. However, for $\Delta\tau/\tau_f^2 = +0.05\ \mathrm{ns}^{-1}$ a peculiar effect is observed. In Fig. 1 A for $\Delta r(\infty) = -0.10$, the anisotropy decreases again after 25 ns, whereas in Fig. 1 C for $\Delta r(\infty) = +0.10$, a rise is calculated after 8 ns.

Experimental

The M13 bacteriophage (wild type) and *Escherichia coli* K37 were a gift of Dr. B. Harmsen, University of Nijmegen. With purified bacteriophage, the phenol-extracted M13 coat protein was prepared according to the procedure of Knippers & Hoffman-Berling (1966). DMPC and DMPG were purchased from Sigma Chemical Co. Octadecyl rhodamine B chloride was obtained from Molecular Probes. Rose bengal, obtained from Eastman Kodak, was further purified by thin layer chromatography.

Reconstitution of M13 coat protein in lipid vesicles was as described previously (Datema et al. 1987 a, b).

Octadecyl rhodamine B was incorporated in the M13 coat protein-mixed DMPC/DMPG (80/20 w/w) lipid system. The probe concentration is 0.03 mol% with respect to the total lipid concentration. The time-resolved single-photon counting instrument as well as the data analysis have been described in detail elsewhere (Van Hoek et al. 1983; Vos et al. 1987; Visser et al. 1988). To determine the temporal pulse response of the system, rose bengal in methanol has been used as the reference compound (Löfroth 1985; Vos et al. 1987; Visser et al. 1988). The temperature, at which the present experiments were performed, is 40 °C, which is above the phase transition temperature (23 °C, Datema et al. 1987 b). At this temperature the lifetime of the reference compound is measured to be 522 ps. After excitation at 580 nm by a dye laser beam, the fluorescence of octadecyl rhodamine B is detected through a cut-off filter (Schott 3 mm RG610) and an interference filter (Balzers B40 613 nm, band width <10 nm). Besides inspection of the weighted residuals and the autocorrelation function, the quality of the fitted fluorescence decay as well as the fitted anisotropy decay is evaluated by the reduced chi square (χ^2) value, the Durbin-Watson parameter and the number of zero passages in the autocorrelation function (O'Connor and Phillips 1984).

Results and discussion

In Fig. 2 the fluorescence decay of octadecyl rhodamine B in a system of bacteriophage M13 coat protein, incorporated in mixed DMPC/DMPG (80/20 w/w) lipid bilayers, is fitted to a single, double as well as triple exponential function. Considering the better fit quality, the fluorescence decay has been fitted to a triple exponential function.

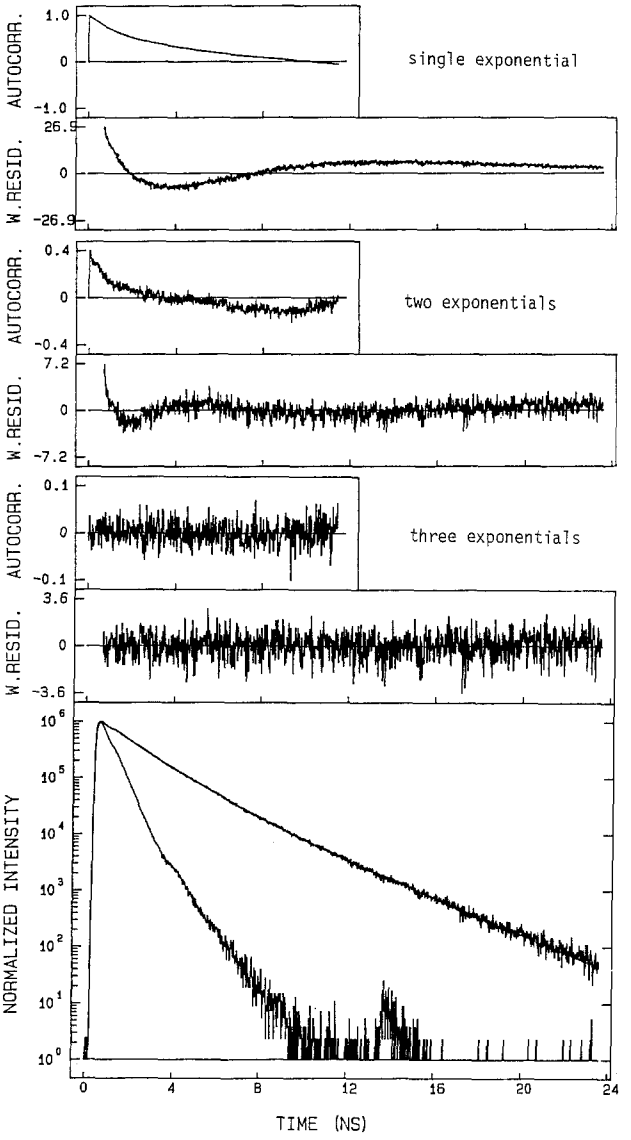

Fig. 2. Analysis of fluorescence decay at 598 nm of octadecyl rhodamine B in the bacteriophage M13 coat protein-mixed DMPC/DMPG (80/20 w/w) lipid bilayer at 40 °C after excitation at 580 nm. The L/P ratio is 5. The fit channel range is from 32 to 1024 (23 ps/channel). The fit parameters are $\alpha_1 = 0.428 \pm 0.037$, $\alpha_2 = 0.427 \pm 0.003$, $\alpha_3 = 0.145 \pm 0.005$, $\tau_1 = 0.040 \pm 0.005$ ns, $\tau_2 = 1.777 \pm 0.017$ ns and $\tau_3 = 3.510 \pm 0.024$ ns, for a triple exponential function. The upper panels also show the weighted residuals and the autocorrelation functions for a double exponential function with $\alpha_1 = 0.670 \pm 0.003$, $\alpha_2 = 0.330 \pm 0.004$, $\tau_1 = 1.455 \pm 0.008$ ns, $\tau_2 = 3.236 \pm 0.010$ ns; and for a single exponential function with $\alpha_1 = 1.000 \pm 0.001$ and $\tau_1 = 2.359 \pm 0.001$ ns. The reduced χ^2 values for these three fits are 1.00, 1.53 and 34.44, respectively. The Durbin-Watson parameters are 2.05, 1.33 and 0.04, respectively. The numbers of zero passages in the autocorrelation function are 264, 60 and 6, respectively

In Table 1, the fluorescence lifetime (τ_j) of each fluorescence component and its corresponding normalized fluorescence contribution (α_j) as well as the average lifetime ($\langle\tau\rangle$) are summarized. As shown in this Table, the average fluorescence lifetime of octadecyl rhodamine B in the protein-lipid system decreases with increasing L/P ratio. From previous measurements of lifetimes, it has been observed that the lifetime of octadecyl rhodamine B and rhodamine B will decrease by more polar environ-

Table 1. The fluorescence decay parameters of octadecyl rhodamine B in bacteriophage M13 coat protein-DMPC/DMPG (80/20 w/w) system, for various L/P ratios at 40 °C. The average lifetime is calculated from

$$\langle\tau\rangle = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i .$$

The χ^2 value and the Durbin-Watson parameter are about 1.0 and 2.0, respectively

L/P	α_1	τ_1 ns	α_2	τ_2 ns	α_3	τ_3 ns	$\langle\tau\rangle$ ns
	±0.04	±0.006	±0.03	±0.02	±0.03	±0.03	
5	0.43	0.040	0.43	1.78	0.14	3.51	2.4
10	0.23	0.038	0.63	1.75	0.14	3.29	2.2
20	0.15	0.034	0.69	1.72	0.16	2.98	2.1
50	0.00	0.000	0.83	1.72	0.18	2.71	2.0
100	−0.13	0.056	0.73	1.58	0.40	2.34	1.9
∞	−0.23	0.085	0.65	1.56	0.57	2.18	1.9

Table 2. The fluorescence anisotropy decay parameters of octadecyl rhodamine B at 40 °C in bacteriophage M13 coat protein-DMPC/DMPG (80/20 w/w) system, for various L/P ratios. The χ^2 value and the Durbin-Watson parameter are about 1.1 and 2.0, respectively

L/P	$(1-C)\beta_f$	ϕ_f ns	$C\beta_b$	ϕ_b ns	$C\{r_b(\infty) - r_f(\infty)\}$	$\tau_f^2/\Delta\tau$ ns	$r_f(\infty)$
	±0.03		±0.02		±0.004	±5.0	
10	0.08	3.02	0.06	2.34	0.078	21.0	0.132
20	0.13	3.02	0.03	2.34	0.052	19.0	0.132
50	0.14	3.02	0.04	2.34	0.031	24.0	0.132
100	0.15	3.02	0.04	2.34	0.016	32.0	0.132
∞	0.21	3.02	–	–	–	–	0.132

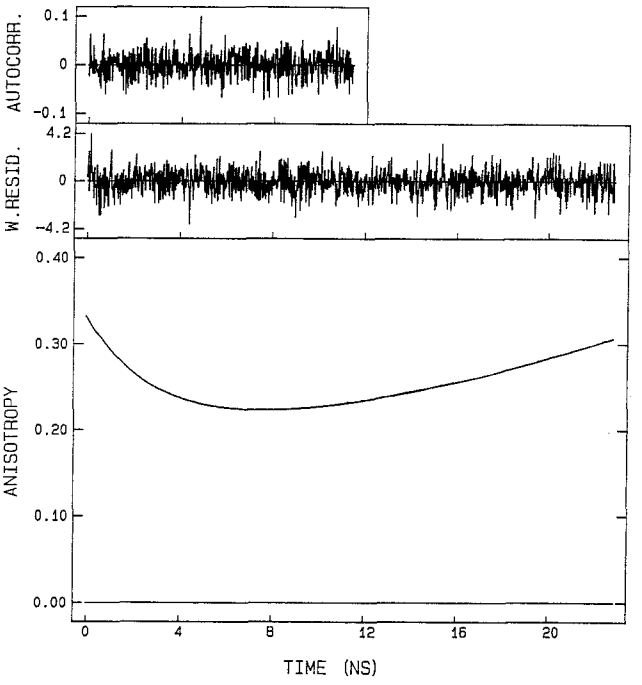

Fig. 3. Fitted fluorescence anisotropy decay at 598 nm of octadecyl rhodamine B in the bacteriophage M13 coat protein-mixed DMPC/DMPG (80/20 w/w) lipid bilayers at 40 °C after excitation at 580 nm. The L/P ratio is 20. The channel range for fitting is from 32 to 1024 (23 ps/channel). The fit parameters are $C\beta_b = 0.03 \pm 0.02$, $(1-C)\beta_f = 0.13 \pm 0.02$, $C\{r_b(\infty) - r_f(\infty)\} = 0.052 \pm 0.003$, $r_f(\infty) = 0.132$, $\phi_b = 2.34$ ns, $\phi_f = 3.02$ ns, $\tau_f^2/\Delta\tau = 19.0 \pm 2.2$ ns. The upper panels show the weighted residuals and the autocorrelation function for the anisotropy reconstructed from the polarized intensity components. The reduced χ^2 value is 1.10 and the Durbin-Watson parameter is 2.02. The numbers of zero passages in the parallel and in the perpendicular intensity components are 234 and 279, respectively

ments (Visser et al. 1988; Khmelnitsky et al. 1989). This may suggest that the aromatic moiety of the protein-bound probe is positioned in an environment of less polarity than that for a free probe in a lipid environment. One should bear in mind that the probe is probably located in the head group region of the lipid molecules. An increase of L/P ratio would be in favour of an increase in population of the probe molecules in the lipid phase and, in turn, the average fluorescence lifetime will become shorter. It should be noted that one of the pre-exponential coefficients is negative mainly in a lipid environment. It may be interpreted as the result of retarded "solvent" relaxation of octadecyl rhodamine B in vesicles, similar to that observed previously for rhodamine B in glycerol (Visser et al. 1988).

Inspection of the average fluorescence lifetimes for different L/P ratios confirms the basic assumption of our theory that due to introduction of proteins into the lipid membrane the relative change in fluorescence lifetime of the probe is small. Therefore, we can apply our approximate theory to analyze the fluorescence anisotropy of the heterogeneous protein-lipid system.

The anisotropy of octadecyl rhodamine B alone in mixed DMPC/DMPG (80/20 w/w) bilayers has been fitted to a single exponential function with a constant term. The correlation time in this system is 3.02 (±0.09) ns. The initial and the residual anisotropies are 0.338 (±0.005) and 0.132 (±0.003), respectively. When the probes are considered to be situated in two distinct environments, the anisotropy for the protein-lipid systems has been fitted to a triple exponential function with a constant, in which, as indicated by (8), some parameters can be directly replaced by those measured from pure lipid bilayers. The correlation time of the bound probe, averaged over experiments at different L/P ratios ranging from 10 to 100, approximately equals 2.34 ns, which is used during fitting. The other anisotropy parameters are listed in Table 2.

A significantly unusual behavior of the time-dependent anisotropies of octadecyl rhodamine B in the M13 coat protein-mixed DMPC/DMPG lipid system, especially in the case of low L/P ratio, can be observed: after an initial decay the anisotropy increases (Fig. 3). The increasing anisotropy results from the positive value of $\Delta\tau$ (see Table 2). This positive value is in agreement with that expected from Table 1: the average fluorescence lifetime of the bound probe is longer than that of the free probe.

Comparing the correlation times of the fitted anisotropy in Table 2 to those in (8), we immediately recognize that the correlation time of protein-bound probes is 2.34 ns, slightly shorter than that of the probes free in the lipid matrix. This may result from an additional electric

potential existing between the positively charged aromatic moiety of octadecyl rhodamine B and negatively charged amino acid residues in the N-terminal region of M13 coat protein. This potential, as a part of the equivalent potential of the Brownian motion, may lead to a change in the diffusion constant and thus in the correlation time (Zannoni et al. 1983).

The relative difference in average lifetimes in the two distinct environments drops approximately from 0.1 to 0.06, as L/P ratio varies from 10 to 100. This is probably caused by the influence of molecular environment on the probe lifetime. Since this influence should depend on the distance between probe and protein, the lifetime may be continuously distributed as function of this distance. The discrete fluorescence lifetimes for the bound and the free probes can be considered to be lifetimes averaged over two probe populations: close to and away from the protein. The increase of the population of free probes, as the result of increasing L/P ratio, leads to a lengthening of the average lifetime for the free probes. At the same time the average lifetime of the probe appearing in the vicinity of the protein will be shortened due to its decreasing population. Considering the fact that the lifetime for the bound probe is longer than that for the free probe, the difference in lifetimes therefore decreases and, in turn, its influence on the anisotropy decay becomes less.

From a comparison of the pre-exponential coefficients of the anisotropy fit to those in (8), we can estimate the relative fluorescence contribution C and the local order parameter for the probe in the two environments. The relative fluorescence contribution is decreased with increasing L/P ratio. For L/P = 10 and 20, it is 62% and 38%, respectively. The values may be compared with those, measured by ESR experiments for a similar protein-lipid system with a fatty acid spin label, where the relative contributions at 30 °C are 72% and 36% for L/P ratio equal to 9 and 18, respectively (Datema et al. 1987b).

The initial anisotropy for the bound probe is estimated to be 0.35, similar to that of the free probe. The orientational order parameter for the bound probes can be estimated as 0.85, while for free probes it is 0.62. From the order parameter, the semiangle of a cone, within which probe wobbles, may be estimated as 22° and 42°, respectively (Heyn 1979; Kinosita et al. 1982). Consequently, the bound probes diffuse somewhat faster and wobble in a smaller space, as compared to the free probes.

Here we have demonstrated that under the assumption of mono-exponentially decaying local anisotropies interesting information can be obtained about fluorescent probes bound to proteins in a lipid-protein system. It should be noted, however, that after simple modification, this approach can be extended to the case that local anisotropies are multiple-exponential (Kinosita et al. 1977; Lipari and Szabo 1980; Zannoni et al. 1983; Van der Meer et al. 1984). Furthermore, in the present approach the microenvironments of the probe are simplified to be binary. In the concept of the local anisotropy, the correlation time of the free probe is the time-average over the population of probes with negligible influence from the protein. The other correlation time is the one, averaged over the remaining population. The same arguments apply for the order parameter, which is the orientational order, averaged over the different probe populations. Further analysis should involve the investigation of the distribution of correlation times and order parameters, as proposed previously for fluorescence lifetimes (Alcala et al. 1987a, b).

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