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LIMITED ROTATIONAL MOTION OF AMPHIPHILIC FLAVINS IN DIPALMITOYLPHOSPHATIDYLCHOLINE VESICLES

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The rotational motion of amphiphilic flavins in dipalmitoyl phospholipid bilayers was investigated with fluorescence anisotropy decay measurements. At temperatures between 10 and 50°C the rotation proved to be anisotropic, which indicated composite motion of both the aliphatic side-chain and the isoalloxazine moiety of the octadecyllumiflavin derivatives. Above the phase transition temperature (crystalline→liquid-crystalline state) the depolarization is complete within the average flavin fluorescence lifetime, implicating unrestricted motion and resulting in a non-ordered microenvironment. In the gel or crystalline state the flavin motion can best be characterized as a limited rotation or librational motion. The fluorescence decay of the flavins is heterogeneous at temperatures between 10 and 50°C, which is explained by assuming nanosecond relaxation of the polar phosphatidyl head-groups around the excited flavin. The lack of a significant cholesterol effect suggests that the isoalloxazine is located at the interphase of the bilayer and not in the hydrocarbon region. The microstructure is fluid-like, not in agreement with a preferred static localization of the flavins in the bilayer.

Introduction

Time-resolved fluorescence anisotropy of intrinsic or extrinsic probes attached to macromolecular systems provides information on the dynamic behaviour of these probes. The decay of the fluorescence anisotropy may depend both on the internal motion of the probe and on the overall motion of the macromolecular substrate. From an analysis of the overall motion the size and shape of the macromolecular object can be estimated. These phenomena and methods have been surveyed in the last decade [1–4], while applications with natural probes have been reported [5–7]. Time-resolved luminescence depolarization has also found wide application in membrane research merely by observation of the rotational behaviour of the incorporated reporter group [8]. Most studies have been carried out with the probe 1,6-diphenyl-1,3,5-hexatriene (DPH), which is embedded in the hydrophobic interior of the membrane (for a review see, for example, Ref. 9). In these partially oriented systems the rotational motion is restricted. Different theories have been developed to account for these hindered rotations [10–13]. An important fact to consider is that the fluorescence anisotropy does not completely decay to zero within the time range of the experiment; it reaches a constant limiting value. Expressions for the order parameter (S) of the probe in the membrane have also been derived [12–15].

Flavins and flavoproteins have been extensively investigated both with steady-state and with time-resolved fluorescence techniques (see, for example, Ref. 16). Spectroscopic properties of flavins embedded in model membranes have also been in-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; OLF, octadecyllumiflavin; DPPC, dipalmitoylphosphatidylcholine.

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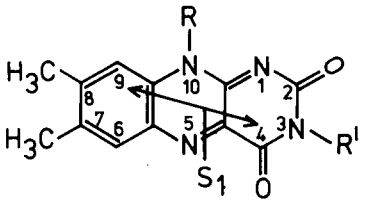

N_3OLF: $R=-CH_3$, $R'=-(CH_2)_{18}-H$

$N_{10}OLF$: $R=-(CH_2)_{18}-H$, $R'=-H$

Fig. 1. Amphiphilic flavins investigated. First electronic transition moment (S_1) is shown. (see Refs. 25 and 33)

vestigated [17–20]. The reason to study flavin models in membranes is closely related to the fact that some flavoproteins are bound to membranes and that some photoreceptors happen to be membrane-bound flavinic pigments [21]. The amphiphilic flavins deserve special attention, since they contain a long aliphatic chain ($-C_{18}H_{37}$), which is buried in the hydrophobic interior of the membrane. The alignment of the aliphatic tail is probably coincident with the membrane normal. Two different flavin molecules were used with the long chain either attached to nitrogen 10 or to nitrogen 3 of the isoalloxazine moieties (Fig. 1). The steady-state fluorescent properties of these amphiphilic flavins in different vesicle systems have been extensively described by Schmidt [17,18]. In this contribution, time-dependent fluorescence anisotropy has been used to investigate the dynamic properties of these flavins in artificial membranes on a (sub)-nanosecond time scale.

The results of the experiments can be summarized. Below the phase transition (gel → liquid crystalline) of the vesicle, the rotational motion of the flavins is restricted: a short and a long rotational correlation time can be discerned. Above the phase transition the rotations appear to be very rapid, and become unrestricted, but remain anisotropic. The situation is slightly more complicated, since the fluorescence decay is non-exponential in all the cases studied. The heterogeneity in lifetimes is probably related to dipolar relaxation of the membrane around the excited-state flavin. These phenomena were recently reviewed by Lakowicz [22].

Materials and Methods

The amphiphillic flavins used in this work were synthesized according to the protocol as given by Michel and Hemmerich [23]. The derivatives are conveniently abbreviated as N(3)OLF and N(10)OLF, indicating the point of attachment of the aliphatic chain. The phospholipid used, L-β,γ-dipalmitoyl-α-phosphatidylcholine (DPPC) purissimum grade, was purchased from Fluka (Basel). Vesicles with or without flavin added were prepared exactly as outlined by Schmidt [17]. Vesicles were centrifuged in a table-top centrifuge (Eppendorf) and thereafter chromatographed on Sephadex G-25 (Pharmacia, Uppsala) with elution buffer 0.05 M sodium phosphate (pH 8.2), all at room temperature and in the dark. The approximate load was one flavin molecule per 100 phospholipid molecules. Vesicles with 33% cholesterol entrapped were prepared according to Kawata et al. [24]. Measurements were carried out directly after preparation of the vesicles.

Fluorescence spectra were run on an Aminco SPF-500 fluorimeter. Steady state fluorescence polarization measurements were carried out on a photon counting instrument (details in Ref. 25), excitation wavelength 450 nm, emission via Schott KV 520. Fluorescence lifetimes and anisotropies were determined with an Ar ion laser single-photon counting system, extensively described elsewhere [16,26–29], excitation wavelength 457.9 nm, emission via Balzers 531 nm interference filter. The raw anisotropy data were directly fitted, without deconvolution from the instrumental response function, since it can be shown from simulations (J. Papenhuijzen and A.J.W.G. Visser, unpublished data) that the time resolution of the system in combination with the measured parameters (lifetimes and correlation times) fully permits such an analysis. The fluorescence lifetimes and rotational correlation times were obtained from a non-linear least-squares analysis [30,31]. The performance of the system was checked with the single-lifetime sample of 3-methyllumiflavin in water (4.60 ± 0.05 ns) [32]. The same sample was also used to obtain the correction factor in the anisotropy analysis (cf. Ref. 26). Weighting factors as derived by Wahl [31] were used for the anisotropy decay data. Usually anisotropy is analyzed

according to a sum of exponentials:

$$r(t) = \sum_{j=1}^{N} r_j \exp(-t/\Phi_j)$$

with $r_0 = \Sigma_{j=1}^{N} r_j$, the initial anisotropy at $t = 0$ and Φ_j the rotational correlation time. In some appropriate cases anisotropy data were analyzed according to:

$$r(t) = (r_0 - r\infty) \exp(-t/\Phi_1) + r_\infty$$

with r_∞ the anisotropy at $t = \infty$, which is in practice equivalent to the duration of experiment (around 40 ns).

Results

Spectral characterization of amphiphilic flavins in DPPC vesicles

Fluorescence spectra of flavin-containing vesicles were similar to those obtained by Schmidt [17]. Also, the degree of polarization of the fluorescence was found of the same order of magnitude for the particular flavin load [18], while KI fluorescence quenching experiments yielded the same Stern-Volmer constant as obtained by Schmidt and Hemmerich [19].

Fluorescence lifetimes of amphiphilic flavins in DPPC vesicles

In all cases the fluorescence decay of flavins in phosphatidylcholine vesicles happens to be heterogeneous. An example is shown in Fig. 2, in which the same fluorescence decay was fitted to a single-, double- and triple-exponential function, respectively. One criterion used is the so-called root-mean-square deviation, RMS (see Ref. 30 for definition) and a sum of three exponentials always yielded the lowest RMS value (Fig. 2). Thus, referring to the single-lifetime standard of 3-methyllumiflavin in water [26,29,32], the membrane introduces a microheterogeneous environment near the flavin. This aspect will be discussed later. The parameters resulting from a triple-exponential function are given in Table I, in which also the average lifetimes are tabulated. Two points are worthy of note. Firstly, the average lifetime and the long lifetime component do not vary much

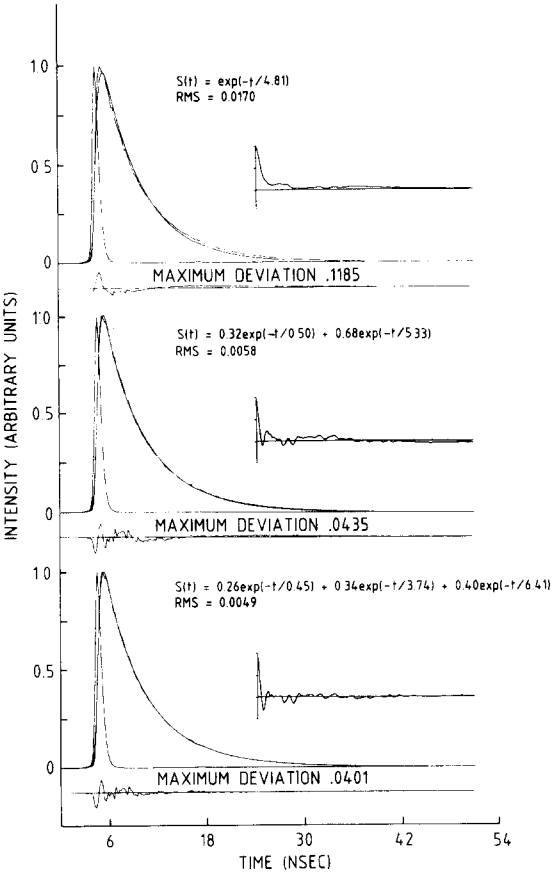

Fig. 2. Fluorescence decay analysis of N(3)OLF in DPPC vesicles at 30°C. The curves show the laser pulse profile and the convoluted fluorescence, normalized to their maximum values. Also shown at the bottom of each panel is the difference between the calculated and experimental response and its maximum value (maximum deviation). The insets show the autocorrelation as a function of the residuals. Top panel: fit to a single exponential function. Middle panel: double-exponential fit. Lower panel: triple-exponential fit.

with increasing temperature, indicating no drastic change in the responsible relaxation processes. Secondly, the average-lifetime and the long-lifetime component of N(3)OLF and N(10)OLF are only slightly different. This is probably due to intrinsic different photochemical properties of N(3)- or N(10)-substituted flavins [33].

Anisotropy decay of amphiphilic flavins in DPPC vesicles

The decay of the fluorescence anisotropy of flavins in DPPC vesicles is heterogeneous. The

TABLE I

FLUORESCENCE LIFETIMES OF AMPHIPHILIC FLAVINS IN PHOSPHATIDYLCHOLINE VESICLES

Results of a fit to a sum of three exponential terms, $\Sigma_{i=1}^{3} \alpha_i = 1$.

	T(°C)	α_1	τ_1(ns)	α_2	τ_2(ns)	α_3	τ_3(ns)	$<\tau>$(ns) [a]
N(3)OLF	10	0.28	0.58	0.24	3.35	0.48	6.46	5.61
	20	0.27	0.50	0.31	3.58	0.42	6.45	5.45
	32	0.26	0.45	0.34	3.74	0.40	6.41	5.38
	41	0.32	0.37	0.28	3.35	0.40	6.35	5.37
	50	0.32	0.36	0.27	3.36	0.41	6.32	5.39
N(10)OLF	10	0.11	0.27	0.16	2.38	0.73	7.03	6.68
	20	0.34	0.39	0.13	3.67	0.53	7.05	6.69
	31	0.26	0.41	0.13	2.58	0.61	6.89	6.44
	40	0.29	0.41	0.10	3.71	0.61	6.99	6.57
	50	0.29	0.37	0.07	3.49	0.64	6.83	6.53

[a] Average lifetime defined by $\sum_{i=1}^{3} \alpha_i \tau_i^2 / \sum_{i=1}^{3} \alpha_i \tau_i$.

obtained correlation times are in contrast to the fluorescence liftetimes very sensitive to variations in temperature. A particular example of an analysis is shown in Fig. 3. The top panel represents a double-exponential function. Both a plot of the weighted residuals and the correlation function of the residuals prove that a decay analysis with two correlation times yields superior results. It can also be observed that the noise in the data increases along the decay. This is due to diminishing fluorescence intensity and it severely limits the usable experimental time scale. The lower panel of Fig. 3 shows an analysis in which one of the correlation times has been fixed to a very long value ($\Phi_2 \rightarrow \infty$). A characteristic feature is the incomplete decay of the anisotropy, it reaches a constant limiting value, r_∞. According to Kinosita et al. [10] the motion of a cylindrically shaped probe in a membrane suspension can be conceived as a diffusive motion within a cone semiangle Θ_0 described by

$$r(t) = (r_0 - r_\infty) \exp(-D_w t / <\sigma>) + r_\infty$$

where r_0 is the limiting anisotropy at $t = 0$, $<\sigma>$ is a constant dependent on Θ_0; Θ_0 and D_w, the wobbling diffusion constant, can be approximated by

$$r_\infty / r_0 = \left[\tfrac{1}{2} \cos\Theta_0 (1 + \cos\Theta_0)\right]^2 \text{ and } \Phi = <\sigma> / D_w$$

This approximation will be discussed later. It is assumed that the emission and absorption transition moments coicide with the symmetry axis of the probe. Under these conditions a general expression for the order parameter, S, of the probe

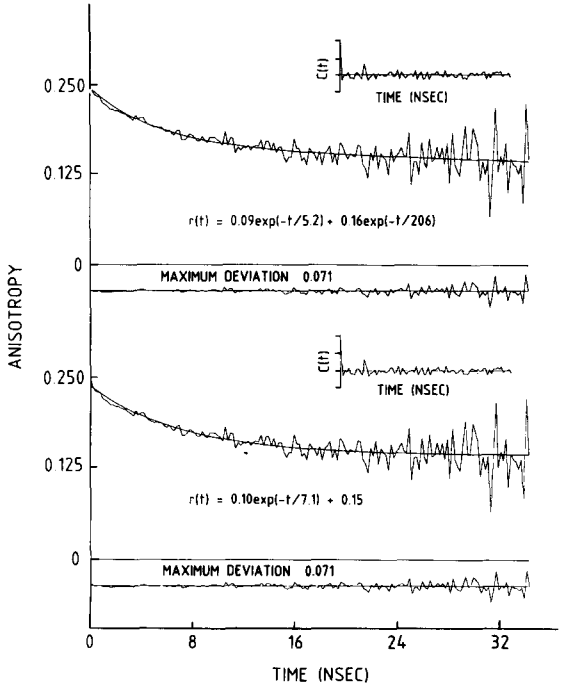

Fig. 3. Fluorescence anisotropy decay analysis of N(3)OLF in DPPC vesicles at 10°C. Top and bottom panels show fits of same experimental data to functions indicated.

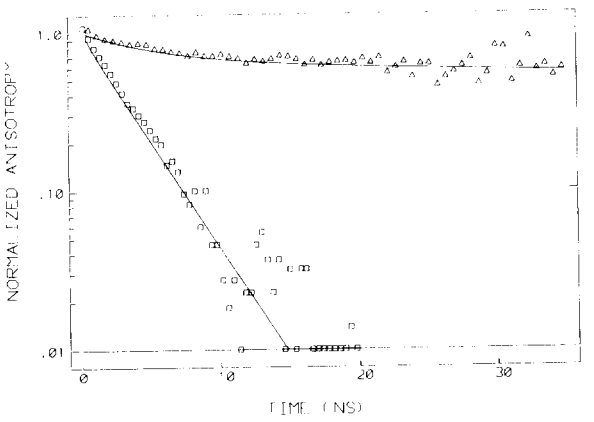

Fig. 4. Experimental and calculated (_____) anisotropy decay of N(3)OLF in DPPC vesicles. △△△ at 10°C, □□□ at 50°C. The parameters of the calculated decays are given in Table II.

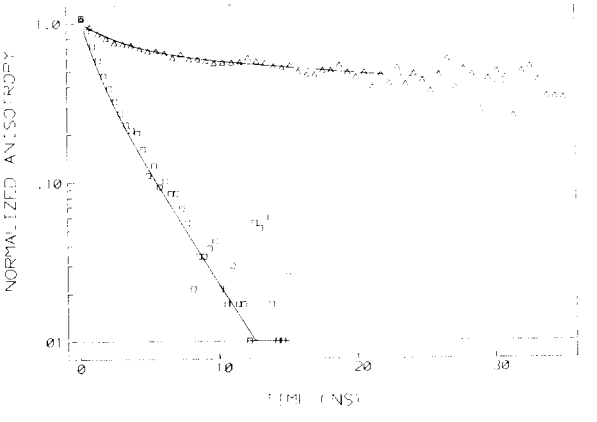

Fig. 5. Experimental and calculated (_____) anisotropy decay of N(10)OLF in DPPC vesicles. △△△ at 10°C, □□□ at 50°C. The parameters of the calculated decays are given in Table II.

in the membrane has been derived [12–14]:

$$r_\infty / r_0 = S^2$$

From the experimental data on the amphiphilic flavins it appeared that such an analysis can be carried out only at temperatures below 30°C. Above this temperature there is complete depolarization, implicating that the wobbling in cone model is not an adequate description of flavin motion. In order to compare the results at different temperatures, plots of the normalized anisotropy ($r(t)/r_0$) were generated with the resulting fits at two temperatures. Fig. 4 shows the data of N(3)OLF at 10 and 50°C, while Fig. 5 gives those of N(10)OLF. At higher temperatures the correlation times become very short owing to increasing motional freedom of the flavin chromophor. All relevant information retrievable from anisotropy decay curves is collected in Table II. The main characteristics can be summarized as follows. There is a progressive decrease in the average correlation times and anisotropies with increasing temperature. There is also an indication of a transition, albeit not sharp, corresponding with a vesicle phase transition from the gel to the liquid crystalline state (T_c). It is also

TABLE II

ANISOTROPY CHARACTERISTICS OF AMPHIPHILIC FLAVINS IN PHOSPHATIDYLCHOLINE VESICLES

$\langle\Phi\rangle$ is the average correlation time [8]: $\langle\Phi\rangle = \Sigma_i \beta_i (\Sigma[\beta_i/\Phi_i])^{-1}$. r_0 is the anisotropy at $t=0$. r_∞ is the anisotropy at $t=35$ ns. $\langle r\rangle$ is the steady-state anisotropy. S is the order parameter [14]: $S=\sqrt{(r_\infty/r_0)}$. Θ_0 is the cone semiangle from: $S=\frac{1}{2}\cos\Theta_0\,(1+\cos\Theta)$.

	T(°C)	β_1	Φ_1(ns)	β_2	Φ_2(ns)	$\langle\Phi\rangle$(ns)	r_0	r_∞	$\langle r\rangle$	S	Θ_0
N(3)OLF	10	0.09	5.2	0.16	206	14	0.25	0.14	0.15	0.74	36
	20	0.06	3.9	0.17	76	13	0.23	0.11	0.14	0.69	39
	32	0.08	2.8	0.16	29	7	0.24	0.05	0.12	0.46	54
	41	0.12	2.7	0.12	9	4	0.24	0	0.04	0	90
	50	0.20	2.5	0.03	5.4	3	0.23	0	0.02	0	90
N(10)OLF	10	0.09	3.1	0.16	64	8	0.25	0.10	0.14	0.63	43
	20	0.12	3.8	0.13	38	7	0.25	0.06	0.12	0.49	52
	31	0.12	2.4	0.12	22	4	0.24	0.03	0.08	0.35	62
	40	0.15	2.7	0.08	19	4	0.23	0.03	0.04	0.29	66
	50	0.11	1.0	0.13	3	2	0.24	0	0.02	0	90

apparent that the initial anisotropy is distinctly lower than the one obtained from 3-methyllumiflavin in a viscous solvent (A.J.W.G. Visser, unpublished data). This probably arises from a rapid initial picosecond depolarization not resolved by the apparatus. Only slight differences between the two compounds N(3)OLF and N(10)OLF can be noticed. The order parameter, S, is low and reveals greater flexibility than in the case of a probe like DPH, which is more deeply buried in the membrane.

The effect of cholesterol on the anisotropy decay of amphiphilic flavins in DPPC vesicles

Even the incorporation of 33% cholesterol in DPPC vesicles hardly changed the dynamic behaviour of the inserted amphiphilic flavins. This is illustrated for N(3)OLF in Fig. 6 at two temperatures. These results are to be distinguished from those of Kawato et al. [24] on DPH in DPPC vesicles, where a profound effect of cholesterol was observed. The fluorescence lifetimes of the flavins were not affected at all.

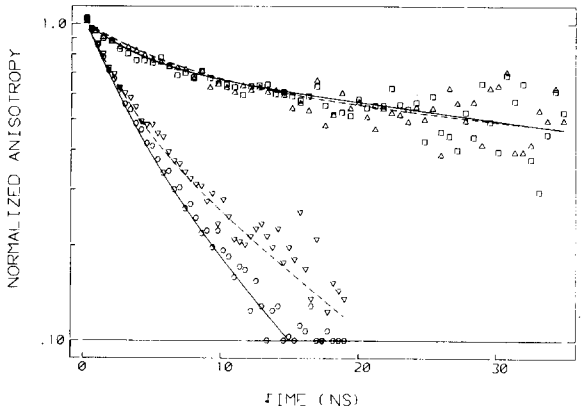

Fig. 6. The effect of cholesterol on the rotational motion of N_3OLF in DPPC vesicles. □□□ without cholesterol at 20°C, △△△ with 33% cholesterol at 20°C, ○○ without cholesterol at 40°C, ▽▽▽ with 33% cholesterol at 40°C; _____ represents calculated decay of flavins in vesicles without cholesterol (parameters listed in Table II), — — — is the fitted function in the presence of cholesterol. The fitted functions fulfill the following equations:
20°C: $r(t)/r_0 = 0.35 \exp(-t/6.4) + 0.65 \exp(-t/100)$
40°C: $r(t)/r_0 = 0.46 \exp(-t/2.6) + 0.54 \exp(-t/12.1)$,
where t is expressed in ns.

Discussion

Dipolar relaxation near the flavins

Owing to improved instrumentation it is clearly apparent that flavins incorporated in a biopolymer matrix undergo characteristic excited-state processes resulting in heterogeneous fluorescence decay. This heterogeneity is not due to inhomogeneous distribution of flavin pigments in the artificial membrane, but should be ascribed to relaxation processes occurring on the same time scale as the lifetime of the excited singlet state. When the flavin is dissolved in water [33,34], relaxation of water molecules is extremely rapid (rate constants of the order of 10^{11} s^{-1}) and fluorescence originates from a solvent relaxed state characterized by a single lifetime. When the relaxation is incomplete, a situation which can be encountered in a solvent such as glycerol, there is a continuous redistribution or reorientation of solvent molecules around the flavin during its excited-state lifetime. At the short wavelength (or high energy) side of the fluorescence spectrum the lifetime is expected to be shorter, while at the red edge of the emission (low energy side) the fluorescence is longer lived corresponding with a relaxed state. Our preliminary investigations of 3-methyllumiflavin in glycerol are in full agreement with this concept [35].

Although the fluorescence wavelength was not systematically varied in this investigation, the different decay rates of the flavin reflect similar relaxation processes in the membrane. The isoalloxazine moiety is located near the phosphatidylcholine and glycerol part of the phosphatidylcholine vesicle, whereas the octadecyl chain is probably anchored along the nonpolar tails. The relaxation of the membrane can then be conceived as the reorientation of the polar head-groups around the excited flavin at a similar rate as the fluorescence process.

Anisotropic motion of the flavins

The results of fluorescence decay of amphiphilic flavins can be interpreted assuming anisotropic motion of the chromophore. The rotational behaviour should, however, be distinguished from that of a probe like DPH, which is expected to be buried in the hydrophobic part of the membrane. The flavin can rotate around the attached aliphatic

chain, which itself can carry out a wobbling-like motion as described for DPH [8,10,11]. This automatically implies a complex motion of the entire molecule, composed of both chain and aromatic contributions. This view is substantiated by the different correlation times measured at temperatures between 10 and 50°C. Below the phase transition temperature, T_c, the chain motion is hindered and the initial rapid decrease in anisotropy can be explained by an independent rapid motion of relatively large amplitude arising from the flavin molecule. Above T_c the depolarization is almost complete, but the decay remains anisotropic and illustrates rapid mobilities of both portions of the molecule. Schmidt [17] has determined microviscosities and rotational relaxation times from the Perrin formula assuming spherical particles. As judged from these results, the assumptions made in that study are not valid and therefore the dynamic behaviour is not adequately described. From Table II, it can be deduced that the cone angle is quite large, indicative of a large-amplitude motion. The equilibrium orientation distribution is therefore not narrowly centered around a distinct average value, but widely spread. The lack of any cholesterol effect proves that the flavin is localized near the polar head-groups and not in the hydrophobic phase. Cholesterol has a strong rigidifying influence on DPH in dipalmitoyl vesicles [24]. The flavins, however, remain as mobile as without cholesterol.

The main assumptions of the wobbling in a cone model are the coincidence of the absorption ($\vec{\mu}_a$) and emission ($\vec{\mu}_e$) transition moments with the symmetry axis of the probe. The following discussion specificly refers to N(3)OLF, since the transition moments ($\vec{\mu}_a$, $\vec{\mu}_e$) point along N(3)-C(8) of isoalloxazine. This vector is able to rotate conically around the octadecyl chain; N(3)OLF is therefore a more suitable model than N(10)OLF. Below T_c the wobbling diffusion coefficient D_w can be estimated following the procedure of Lipari and Szabo [12]. Thus the area under the function $(r(t) - r_\infty)/r_0$ yields $\tau_{eff}(1 - A_\infty)$, where τ_{eff} is an effective correlation time and $A_\infty = r_\infty/r_0$. From these values the wobbling diffusion coefficient D_w can be calculated (using eqn. 24 of Ref. 12). For small cone angles ($\Theta_0 < 30°$), D_w can be approximated by $D_w \approx 7\Theta_0^2/24\tau_{eff}$ (Θ_0 in radians). In Fig. 7 some

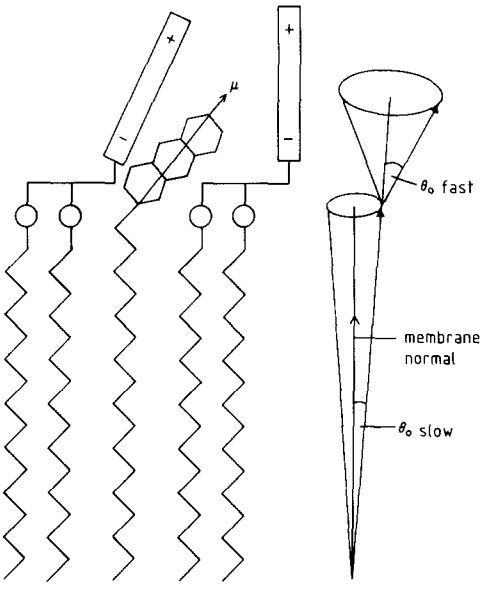

Fig. 7. Schematic representation of membrane-bound flavins. Only half of the bilayer is shown. The flavin N(3)OLF is depicted as a triple hexagon. The polar head groups are drawn as rectangles. The transition moment μ of the flavin is the vector which rotates conically ('wobbles') with semiangle Θ_0 around the long alifatic chain. The long chain is coincident with the membrane symmetry axis. Both vectors are sketched separately to indicate the type of rotational motion below the phase transition temperature. Both isoalloxazine (Θ_0 fast) and the chain (Θ_0 slow) perform a restricted rotation. At higher temperatures, Θ_0 increases and the rotational potential energy barrier is lowered. At a certain temperature above the phase transition temperature Θ_0 can become so large that the flavin rotates nearly unrestrictedly. The anisotropy data at 10°C yielded (approximately from $D_w = 0.292\Theta_0^2/\tau_{eff}$) $D_w = 1.7 \cdot 10^7$ s^{-1}; $\Theta_0 = 35°$. At 32°C $D_w = 3 \cdot 10^7$ s^{-1} and $\Theta_0 = 55°$ is obtained.

situations are schematically depicted, together with estimated values of D_w and Θ_0. Near and above T_c the cone angle becomes so large that the flavin approaches unrestricted motion.

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