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On the Redox Reactions and Accessibility of Amphiphilic Flavins in Artificial Membrane Vesicles

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Summary. (Photo-)redox reactions of different amphiphilic flavins bound to artificial membrane vesicles made from three different, saturated phospholipids have been investigated and compared with those of isotropically dissolved flavin. By means of C_{18}-hydrocarbon chains, substituted at different positions, the flavin nucleus can be specifically oriented within the membrane, thereby imposing sterically anisotropic environments, which are liable to control flavin (photo-) chemistry. A spectrophotometric setup was designed, permitting photoreduction of flavin and its simultaneous control by fluorescence. The characteristic temperature dependency of the (vesicle-bound) flavin photoreduction by external and internal photosubstrates, as studied for the different lipid/flavin systems, is explained by the displacement of the flavin nucleus from the area of the polar head groups of the lipid into the more hydrophobic parts of the membrane upon phase transition (gel→liquid crystalline). Evidence is presented that this flavin displacement is correlated with the pre-phase transition rather than with the main phase transition, supporting a former hypothesis of the structural nature of the pre-phase transitions. The transport of redox equivalents across flavin-charged membranes is discussed. The accessibility of vesicle-bound flavins by a variety of exogeneous ions (H^+, Cs^+, EDTA, NTA, BH_3CN^-, I^-, N_3^-) is explored as a function of temperature, i.e., membrane phase which, in turn, appears to control the permeability of the lipid/water interface. Therefore, it appears indispensable to include the interface as a separate structural entity into any theory on membrane transport.

In the preceding paper of this series [23] data had been presented describing the preparation and photophysical properties of vesicle-bound flavins. On this basis we investigate in the present work several photochemical features of these systems with the ultimate goals:

1) to learn more about the biologically relevant "anisotropic flavin chemistry," which differs – as our results show – essentially from the commonly studied and fairly well-understood "isotropic flavin chemistry," i.e., the chemistry in solution (cf. [2, 9]), and

2) to learn more about the *primary* reactions of physiological blue-light action, a widespread phenomenon throughout the living world [24].

As minimum model system for the blue-light receptor and anisotropic flavin chemistry, three different "amphiflavins" bearing C_{18}-hydrocarbon chains at positions 3, 7 and 10 have been synthesized ("AFl 3", "AFl 7", AFl 10"[1], [21]) and anchored within artificial vesicle membranes made from three different lecithins. Thereby, we hope to expose the various "active centers" of the flavin [10, 13, 14] optionally, which would allow for a new correlation of reaction types and mechanisms.

The main results described in a first report [23] can be summarized as follows: The different amphiphilic flavins ("amphiflavins," AFl 3, 7 and 10) bind to vesicle membranes made from synthetic lecithins with a maximal molar flavin/lipid ratio of 1–2/100. The diameter of the vesicles is about 450 Å, each carrying roughly 200 flavin molecules, with a mean interflavin distance of 50 to 70 Å. The flavin nuclei are mainly confined to the polar head group and membrane/water interface area. Upon membrane phase transitions (gel→liquid crystalline) the nuclei sink deeper into the more hydrophobic (and more

[1] *Abbreviations.* DML: L-β, γ dimyristoyl-α-lecithin; DPL: L-β, γ dipalmitoyl-α-lecithin; DSL: L-β, γ distearoyl-α-lecithin; AFl 3: 7,8,10-trimethyl-3-octadecyl-isoalloxazin; AFl 7: 3,8,10-trimethyl-7-octadecyl-isoalloxazin; AFl 10: 3,7,8-trimethyl-10-octadecyl-isoalloxazin; EDTA: Ethylenediaminetetraacetic acid; NTA: Nitrilotriacetic acid; T_c: Phase transition temperature.

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fluid) part of the membrane, as reflected by up to 76% increase in fluorescence quantum yield (the flavin fluorescence quantum yield increases with decreasing polarity of the solvent), a fivefold decrease in viscosity of the flavin microenvironment (from about 150 to 30 cP; *cf.* the viscosity of water is 1.0 cP) and a concomitant decrease in the rotational relaxation time from more than 50 nsec in the gel state to about 15 nsec in the liquid crystalline state of the membrane, regardless of the specific amphiflavin (i.e., mode of anchoring) used.

Based on fluorescence analysis, an averaged *Stoke*-diameter of the flavin chromophore of 10.2 Å was determined. Surprisingly, the rotational motility of the chromophore is not significantly hindered by the aliphatic chain attached. Whenever the membrane is in the *liquid crystalline* state, the rotational relaxation of the chromophore is effectively slowed down by the exogenous bulk viscosity (glycerol) in a *biphasic* fashion; however, in the *gel* state of the membrane such an influence is not observed at all. This feature is attributed to properties of the interface, which is further substantiated below.

Materials and Methods

Chemicals

A detailed description of synthesis of all flavins used is given in the accompanying paper [21]. The phospholipids L-β, γ dimyristoyl-α-lecithin (DML, N 42803), L-β, γ dipalmitoyl-α-lecithin (DPL,N 42556), L-β, γ distearoyl-α-lecithin (DSL,N 43698), puriss. and tributyrin (glycerol tributyrate, 91010), Ca-phosphorylcholin (B 768569), potassium iodide (5043), sodium azide (6699) and octadecan (74705) were purchased from Fluka Buchs, Neu-Ulm. Control experiments were performed with the same lipids but from another source (Sigma: DML,P 5141; DPL,P 6138; DSL,P 1138). The preparation of the flavin-loaded vesicles has been previously described in detail [23]. The phase transition temperature (T_c), at which the gel (≡ *crystalline*) phase is transformed into the *liquid crystalline* phase, depends strongly on the length of the fatty acid residues of the lecithin employed: DML≙ 23 °C; DPL≙41 °C; DSL≙58 °C. Sodiumcyanoborohydride (15.615–9) was obtained from EGA-Chemie, Steinheim, and nitrilotriacetic acid (NTA,NI 068) from Schuchardt, Muenchen. Ethylenediaminetetraacetic acid (EDTA) was obtained from Merck, Darmstadt, either solubilized (0.1 M Titriplex III, 8431) or in solid form (Titriplex III, 8418). The experiments were generally performed in 0.01 M phosphate buffer at pH 8.0, containing 0.01 M NaCl. The pH for the investigation of the pH dependence of the fluorescence quantum efficiency was adjusted as follows (0.01 M): pH 0/1, HCl; pH 2/3; sulfate; pH 4/5, acetate; pH 6/7/8, phosphate; pH 9/10/11, borate; pH 12, phosphate; pH 13, NaOH/KCl. These suspension media are photochemically inert. The experiments were performed under anaerobic conditions, which were achieved by extensive deaeration of the sample with argon.

Spectroscopy

A simple setup using a vertical light pass was used which permits effective photoreduction of flavin and its simultaneous measurement on the basis of fluorescence [25]. An anaerobic steel cuvette of 1 cm inner diameter (filled up with 1.57 ml sample suspension yielding an effective sample thickness of 2 cm), with air-tight quartz windows at top and bottom is temperature controlled within ±0.5 °C by circulation-thermostated water through a copper cell holder. The effective flavin concentration employed must not exceed 4 μM (A=0.05 at 450 nm) to ensure proportionality of flavin concentration and fluorescence quantum efficiency (calibration curve not shown here). The sample is irradiated with broad blue light of 180 W/m^2 at 450 nm, as obtained from a 100 W halogen lamp activated by a stabilized power supply. In addition to the 450-nm filter we use the "inverse" cutoff filter FITC-6 by Balzers, Liechtenstein, which cuts off wavelengths *above* 510 nm, efficiently diminishing stray light. The fluorescence light is selectively filtered by a set of three blocking filters cutting off below 515 nm and (in order to decrease the flourescence of the cutoff filters themselves) an interference filter of 546 nm (Balzers) closest to the sample, excluding the exciting blue-light efficiently, and detected by an "end-on"-photomultiplier (EMI, 9658 B). The signal is fed via a homemade offset amplifier to a Hewlett Packard strip chart recorder (7101 B). Prior to each set of experiments the system was calibrated with a "standard reaction," i.e., the (anaerobic) photoreduction of an isotropic solution of 4 μM 3-methyl-lumiflavin by 0.01 M EDTA, pH=8.

Following standard procedure, the quantum efficiency of flavin photoreduction was calculated on the basis of the *initial* slope of the kinetics (fluorescence *vs.* time of irradiation). Due to this procedure, only the *fast* component of any biphasic photoreduction kinetics is monitored which, in turn, we assume (see below) to reflect mainly properties of the membrane/water interface; this fast portion of the biphasic kinetics can be determined with an uncertainty of ± 10%; the slower component is poorly reproducible in quantitative terms and only referred to as being slow (min). The mean value and standard error of the mean were calculated for 5–10 parallel kinetics for each condition (Texas Instruments, TI 59, program "1"). Fluorescence quenching experiments, as depicted in Fig. 12 and Table 3, were performed in 1 cm standard fluorescence cuvettes using the (noncorrecting) Perkin Elmer fluorimeter MPF-3. Excitation was at 450 nm, and the height of the emission peak at 525 nm without quencher present was normalized to 100%. The quenching capability was quantified on the basis of the *Stern Volmer* equation: $k_q=(\varphi^0/\varphi-1)/(\tau^0+[\varphi])$ with k_q, quenching rate constant; φ^0, quantum efficiency of flavin fluorescence; φ, the same with quencher present; τ^0, averaged flavin fluorescence lifetime without quencher (2.3×10^{-9}sec) [23]; Q, quencher. The fluorescence lifetime τ^0 of flavins never decreases more than by a factor of 0.6 when transferred from isotropic, aqueous environment (buffer) into vesicle membranes, as we conclude from spectral properties [23]. For the evaluation of k_q for the different quenchers and different membrane phases investigated, τ^0 can therefore – with good approximation – be assumed to be constant.

Fully corrected fluorescence excitation and emission spectra of membrane-bound flavin have been recorded with the JY 3 CI-fluorimeter (Jobin Yvon), on-line with a Hewlett Packard 9825A desk-top computer.

Absorption spectra were measured at room temperature and at liquid-nitrogen temperature with a single-beam spectrophotometer on-line with a small computer (Nicolet 1174), as introduced by Butler and Hopkins [4]. The measurements at liquid nitrogen temperature were made as described by Butler [3]. The arrangement of common double-beam spectrometers requires samples which show absolutely no scattering in order to obtain true absorption spectra, otherwise "extinction spectra" (=absorption+scattering) will be obtained. We overcame this problem by an "end-on" arrangement of the photomultiplier with a large diameter of 2 inches of the light-sensitive surface (EMI 9658 B), mounted as closely

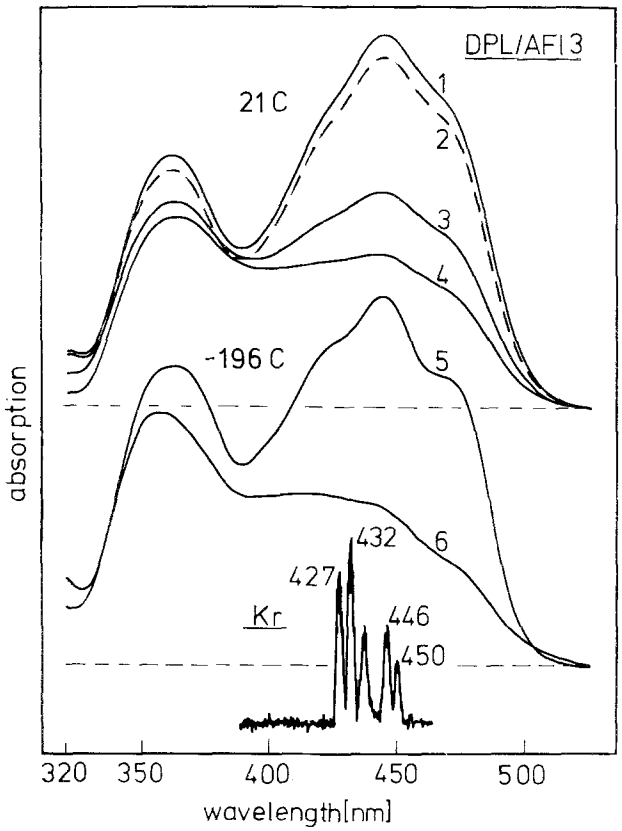

Fig. 1. Absorption spectra of the different redox states of AFl 3 bound to DPL vesicles. *Top,* room temperature: curve 1, fully oxidized form; curve 3, after 5 min; curve 4, after 20 min blue light irradiation, in the presence of 0.1 M EDTA. Curve 2, after reoxidation with atmospheric oxygen. *Bottom,* liquid nitrogen temperature: curve 5, fully oxidized form; curve 6, after 10 min blue light irradiation in the presence of 0.1 M EDTA prior to freezing. A reliable wavelength calibration is given by the blue krypton lines. The spectra are directly plotted by the computer

as possible to the sample (1.2 cm), thereby collecting most of the scattered light (room angle of 1 π compared to about 0.001 π of conventional double-beam spectrometers). This ensures that even in highly light-scattering samples (as vesicle-suspensions) *true* absorption spectra can be measured, since light loss by scattering is largely avoided.

Because of the zwitterionic structure and high charge of this substrate, EDTA-dependent photoreduction of flavin is strictly confined to the aqueous phase (*cf.* [26]). Whereas the oxidized flavin exhibits a strong fluorescence emission (quantum efficiency ranging from 28 to 68%, depending on solvent polarity [23]), the reduced form is nonfluorescent. True absorption spectra of AFl 3 bound to DPL vesicles are shown in Fig. 1 for different redox states of the flavin, at 21 and −196 °C (liquid nitrogen). A completely reduced form is not shown, since its formation requires much actinic light irradiation, causing concomitant photolysis (*see* below). Note that at both temperatures (at −196 °C the extinction is largely due to scattering) the spectrum of reduced flavin decreases sharply below 350 nm – in contrast to commonly reported spectra [5, 11, 29] – a consequence of the "end-on" multiplier arrangement. The vibration splitting of the spectrum of the oxidized form indicates a localization of the chromphore in the polar head group range of the membrane, with a dielectric constant of about $\varepsilon = 30$.

Results

Corrected Fluorescence Spectra of AFl 3 Bound to DPL-Vesicles

In our last publication [23] we stated qualitatively that "the fluorescence emission peak of membrane-bound flavins is shifted hypsochromically by a few nanometers upon phase transition (gel→liquid crystalline)." Using latest equipment (*see* Materials and Methods) we are now able to present fully corrected excitation- and emission spectra, as a function of temperature (i.e., phase) (Fig. 2). It turns out that the hypsochromic shift of the fluorescence emission peak is much more pronounced than that of the *S2*-"absorption" peak (since the excitation spectrum is corrected, both can be assumed to the essentially identical; however, a closer inspection shows decreasing peak heights of the 360, 270 and 230 nm fluorescence excitation peaks, relative to absorption; W. Schmidt, *unpublished*). The peak position of fluorescence emission is also included in Fig. 6, depicting the quantum efficiencies of fluorescence and photoreduction, as well as the autoxidation rate of membrane-bound flavin, as a function of temperature.

Photoreduction of Vesicle-Bound Flavins

Some typical photoreduction kinetics are depicted in Fig.3. 3-Me-lumiflavin in the presence of 0.1 M EDTA is photoreduced anaerobically in a fast first-order reaction (under the present conditions within about 0.5 min) which was used as reference (*see* Materials and Methods). The photoreduction of *membrane-bound* flavins depends strongly on a variety of parameters. At 45 °C (i.e., above melting temperature T_c), with no exogenous donor present, only little fluorescence decrease takes place (about 1%/min), being not reversible by oxygen which is indicative of flavin *photolysis*. However, under the same conditions, but below T_c, all amphiflavins show appreciable (with oxygen reversible) photoreduction by some *internal* donor which is most likely part of the lipid itself (lipids of a different manufacturer show the same effect; contamination is largely excluded by thin-layer chromatography). This holds true for all other vesicle/flavin combinations, as well. In the presence of 0.25 M EDTA as external donor, photoreduction occurs below *and* above phase transition (with both internal *and* external donors contributing below T_c, *see* below).

The ease of photoreduction is visualized more clearly when the initial slope of the kinetics is converted into quantum efficiency Φ of photoreduction. On this basis, Φ has been more thoroughly investigated as a function of (i) the membrane phase, of (ii)

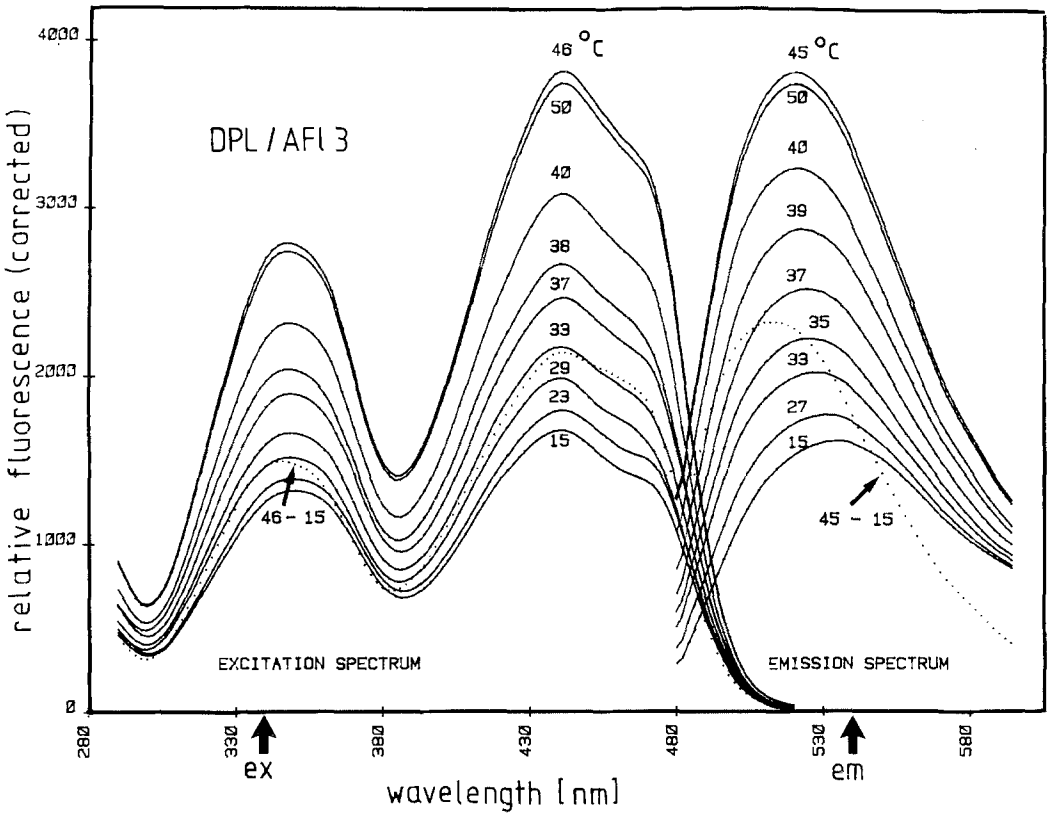

Fig. 2. Corrected fluorescence excitation and emission spectra of AFl 3 bound to DPL vesicles as function of temperature (i.e., membrane phase), as indicated. The highest peaks are adjusted to the same height. The effective flavin concentration is 4 μM (A=0.05 at 450 nm). Respective excitation and emission wavelengths used to monitor the emission or excitation spectra are marked by arrows on the abscissa. Dotted curves represent difference spectra, as indicated. Both excitation and emission monochromator slits are set to 4 nm. Temperature is controlled within ±0.5 °C by circulating thermostated water through a copper cell holder and measured by a thermocouple dipped into the vesicle suspension. The spectra are plotted by the computer

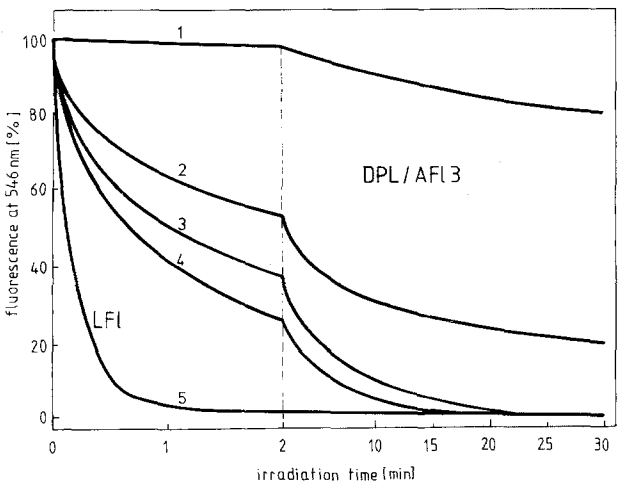

Fig. 3. Actual photoreduction kinetics of aqueous lumiflavin and AFl 3 bound to DPL vesicles, as obtained with the set-up described in the text. Curve 1, 45 °C, no EDTA; curve 2, 21 °C, no EDTA; curve 3, 45 °C, 0.25 M EDTA; curve 4, 21 °C, 0.25 M EDTA; curve 5, lumiflavin 21 °C, 0.1 M EDTA. The kinetics are normalized to 100%

the concentration of various exogenous electron donors and (iii) of the specific mode of anchoring of the flavin within the membrane (accomplished by the specific amphiflavin used), as illustrated in Figs. 4 and 5. These curves suggest that the external substrates have easier access to the flavin nucleus when the membrane is in the *gel* rather than in the *liquid crystalline* state. Furthermore, EDTA and NTA as $1e^-$-transferring substrates show quite similar dependencies as BH_2CN^- as $2e^-$-donor [12]. However, the patterns exhibited in Figs. 4 and 5 appear to be more sensitive to the specific amphiflavin (requivalent to the mode of anchoring) than to the specific donor used. For example (Fig. 4*A*), at high concentrations of EDTA ($>10^{-2}$ M) and above T_c, AFl 3 is efficiently photoreduced ($\Phi=0.04$) exclusively by the external donor (a property utilized below for kinetic investigations), which is not the case with AFl 10 (Fig. 4*B*) and AFl (Fig. 4*C*). Moreover, AFl 7 is about four times less effectively photoreduced by both external and internal donors (Fig. 4*C*). For comparison with

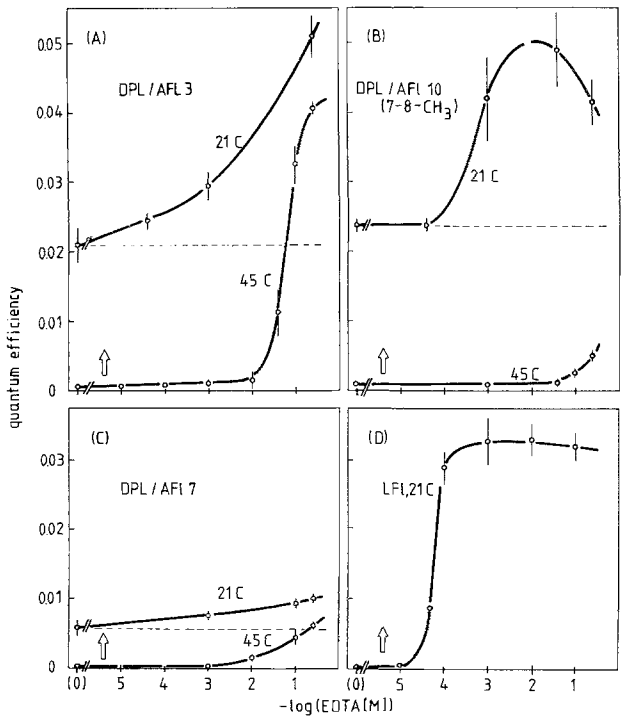

Fig. 4. Quantum efficiencies of photoreduction of different DPL-vesicle-bound flavins, compared with isotropically dissolved lumiflavin, as a function of EDTA concentration and temperature (i.e., vesicle phase). The flavin concentration of 4 μM, as used in all cases, is indicated by the arrows. The dashed lines mark the photoreduction due to an intrinsic donor

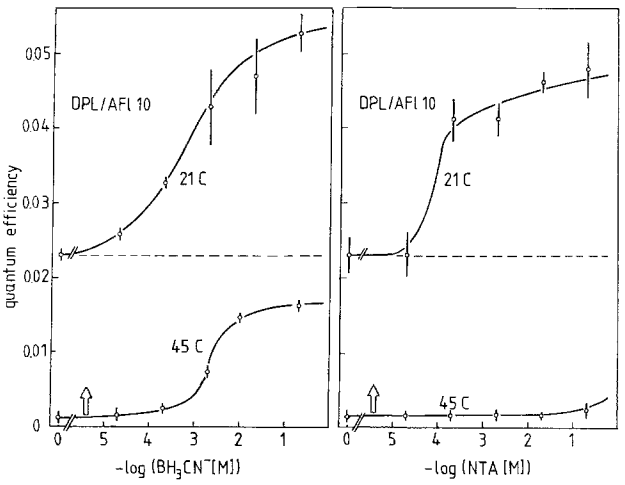

Fig. 5. Similar plot as in Fig. 4, except that sodiumcyanoborohydride (left) and nitriloacetic acid (right) were used as electron donors

the isotropic case the photoreduction of free 3-Me-lumiflavin (Fig. 4*D*) saturates already at a 100-fold excess of EDTA (near $\Phi = 0.03$), indicating that factors other than the EDTA-flavin collision rate are now limiting the photoreduction kinetics. These data

Table 1. Quantum efficiency Φ of flavin photoreduction as a function of various parameters[a]

Flavin, solvent, system	Substrate	$10^5 * \Phi$	T[°C]
Isotropic			
3-Me-LFl, H_2O (55 M)	–	20	20
3-Me-LFl, H_2O (55 M)	EDTA (10^{-3} M)	3000	20
3-Me-LFl, H_2O (3.4 M)	Ca-phosphoryl-cholin (0.4 M)	20	20
AFl 10, CCl_4 (10.3 M)	–	32	20
AFl 10, CCl_4 (10.3 M)	DPL (0.04 M)	46	20
AFl 10, octadecan (3.0 M)	–	420	30
AFl 10, tributyrin (3.4 M)	–	210	20
Anisotropic			
DPL/AFl 3	–	2100	21
	–	20	45
	EDTA (0.25 M)	5000	21
	EDTA (0.25 M)	4000	45
DPL/AFl 10	–	2300	21
	–	20	45
	EDTA	4000	21
	EDTA	500	45
	BH_3CN^- (0.25 M)	5200	21
	BH_3CN^- (0.25 M)	1600	45
	NTA	4800	21
	NTA	200	45
DPL/AFl 7	–	600	21
	–	20	45
	EDTA (0.25 M)	1000	21
	EDTA (0.25 M)	600	45

[a] The vesicle systems have been investigated below (20 °C) and above phase (45 °C) transition temperature of DPL. For comparison, the local lipid concentration as seen by the membrane-bound flavin is as high as 3 to 5 M.

are quantitatively listed in Table 1. The quantum efficiency of 0.03, which we determined for the isotropic system (Fig. 4), is nicely consistent with the value of 0.05 as obtained by Radda and Calvin [22]. In the homogeneous model case we have to account, furthermore, for photoreactions between excited and ground state Fl_{ox} [12]. This is not the case in the amphiflavin vesicle system, where the average distance between two flavin moieties is 50 Å, and the flavin-flavin interaction is decreased by a factor of about 25, compared to the isotropic case (W. Schmidt, *unpublished*). In fact, "interflavin" photoreaction probability increases with temperature, while in the present case of amphiflavin vesicles the internal photoreduction characteristically decreases with temperature.

Nature of the Internal Donor

To obtain a more detailed picture of the flavin photoreduction by the membrane-inherent (internal) elec-

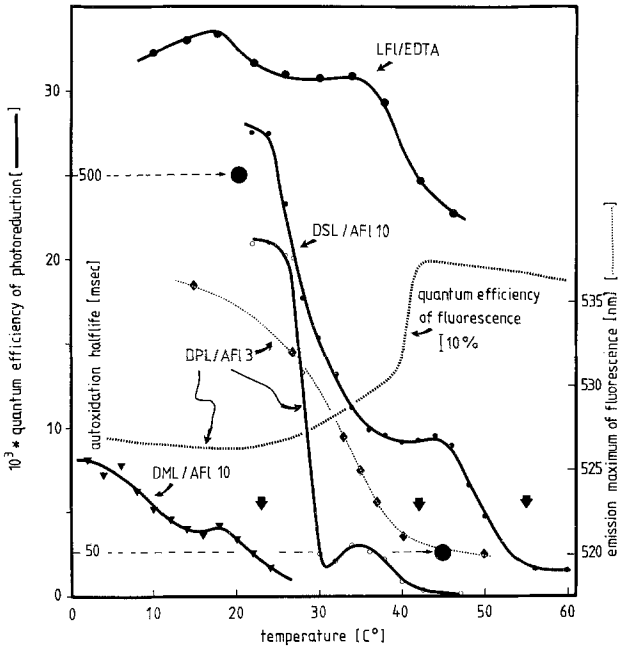

Fig. 6. Quantum efficiencies of photoreduction as a function of temperature, i.e., vesicle phase, as measured for a few flavin/vesicle systems (solid lines). No exogenous electron donor was added. The quantum efficiency already decreases far below the corresponding phase transition (indicated by the arrows). As a comparison, the temperature dependency of isotropically dissolved lumiflavin in buffer, in the presence of 0.01 M EDTA is included (top). For further correlation the temperature dependency of fluorescence quantum efficiency of DPL/AFl 3 (former publication [23]), as well as of the emission peak maximum (Fig. 2) are included. The two big dots mark the autoxidation halflives of reduced AFl 10 bound to DPL vesicles in the *gel* and in the *liquid crystalline* state, respectively (Fig. 10)

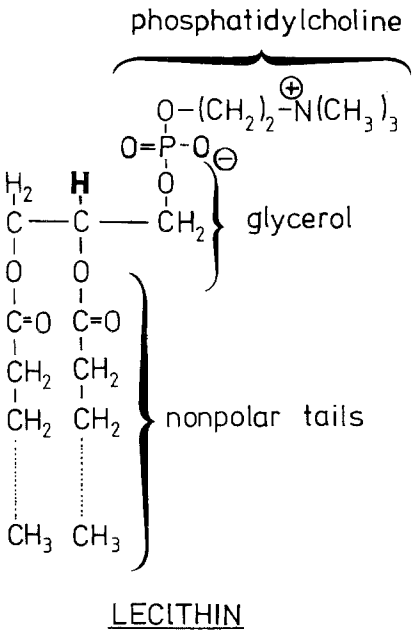

Fig. 7. The structure of the lecithin molecule, as composed by three different molecular entities. The marked hydrogen atom is most likely the electron donating position (*cf.* text)

tron donor as a function of the membrane phase, we measured the photoreduction efficiency as a function of temperature for several membrane/flavin systems, without external donors added. Surprisingly complicated patterns are obtained, as depicted in Fig. 6. The arrows indicate the known melting points (T_c) of DML, DPL, DSL vesicles (*cf.* [23]). For comparison, the temperature dependency of aqueous (isotropic) lumiflavin photoreduction by a saturating EDTA concentration (0.01 M) is included. The membrane-bound flavins already exhibit a strong change of photoreduction speed with temperature *far below* phase transition and a smaller "dip" in between.

The main phase transition is preceded by a so-called "prephase transition," as has been detected independently by differential scanning calorimetry [15, 27] (DPL: pre-phase transition, $\Delta H = 2.3 \pm 0.2$ kcal/mole; main phase transition, $\Delta H = 9.69 \pm 0.06$ kcal/mole). These pre-phase transitions are also reflected in the fluorescence dependency of vesicle-bound flavins on temperature (*cf.* Fig. 4 in [23]). Even if the physico-chemical interpretation of the pre-phase transition is not yet clear [27], it is clearly correlated with the main change in quantum efficiency of photoreduction of the membrane-bound flavins by the internal electron donor. The smaller optima ("dips") in the curves of Fig. 6 coincide approximately with the beginning of the main phase transitions (*note*: it had been shown by *Rayleigh* scattering before [23] that the temperature of the main phase transition, T_c, is *not* altered by the flavin load of the vesicles).

Which part of the lecithin molecule (Fig. 7) acts as the internal donor? A trivial lipid contamination is not only excluded by thin-layer chromatography but also by the fact that the internal donor is only accessible to the flavin in the *gel* phase of the membrane. As "model" molecules, representing the different microenvironments within the membrane (*cf.* Figs. 7, 13), we employed octadecan (for the nonpolar tails), tributyrin (for the glycerol backbone), and phosphorylcholin (for the polar head group) as potential donors in isotropic solution. The quantitative results (uncertainty ranges between 5 to 10%) are depicted in Table 1, together with the vesicle-based data. Octadecan and tributyrin could be used as pure solvents and are fairly effective flavin photosubstrates, Ca-phosphorylcholin, on the other hand, in saturated aqueous solution (0.4 M) shows no measurable effect. (*Note:* the *intrinsic* lipid concentration in the case of vesicles is comparable with the isotropic concentration of octadecan and tributyrin (3 to 5 M); nevertheless, these model substrates are by a factor 5 to 10 times less effective under *isotropic* conditions.)

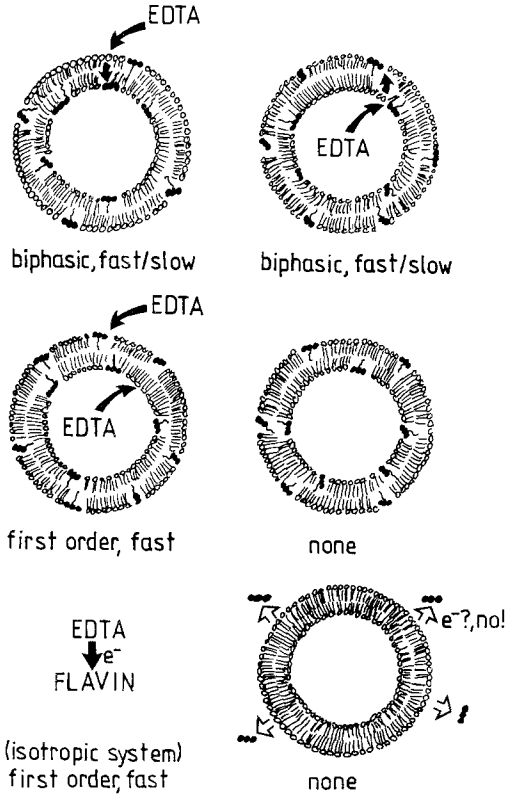

Fig. 8. Qualitative course of photoreduction kinetics of vesicle-bound and free flavins obtained for different arrangements, as visualized. The lipid molecules are sketched as open circles with dashes, the flavin nuclei as black triple dots

Inside/Outside Problem

In our preparations [23] the amphiflavins will be distributed with their nuclei to the outside *and* inside membrane/water interface region of the vesicle. The following experimental cases have been checked as visualized in Fig. 8:

When *only* the external donor EDTA is capable of photoreducing the flavin, we would expect *composite* kinetics reflecting a *fast* photoreduction of outer flavin followed by a *slow* photoreduction of the inner flavin. This was verified for the DPL/AFl 3 system at 45 °C and 0.25 M EDTA (*cf.* Fig. 4*A*), as depicted in Fig. 9*A*. The kinetics can be resolved into two approximately first-order kinetics with halflives of about 15 sec respectively more than 3 min and a corresponding concentration ratio of approximately 1:1, as determined by the intercept in the half logarithmic plot (dashed lines, Fig. 9*A*). For comparison, the photoreduction of free 3-Me-lumiflavin at saturating EDTA concentration proceeds with a monophasic first-order kinetic and a halflife of 9 sec (Fig. 9*B*). The opposite case, i.e., when only the EDTA dissolved in the inner vesicle volume serves as flavin photosubstrate, also reveals a biphasic kinetics similar to that in Fig. 9*A* (Table 2). However, if EDTA was present inside *and* outside the vesicle, the membrane-bound flavin was photoreduced with a single, fast

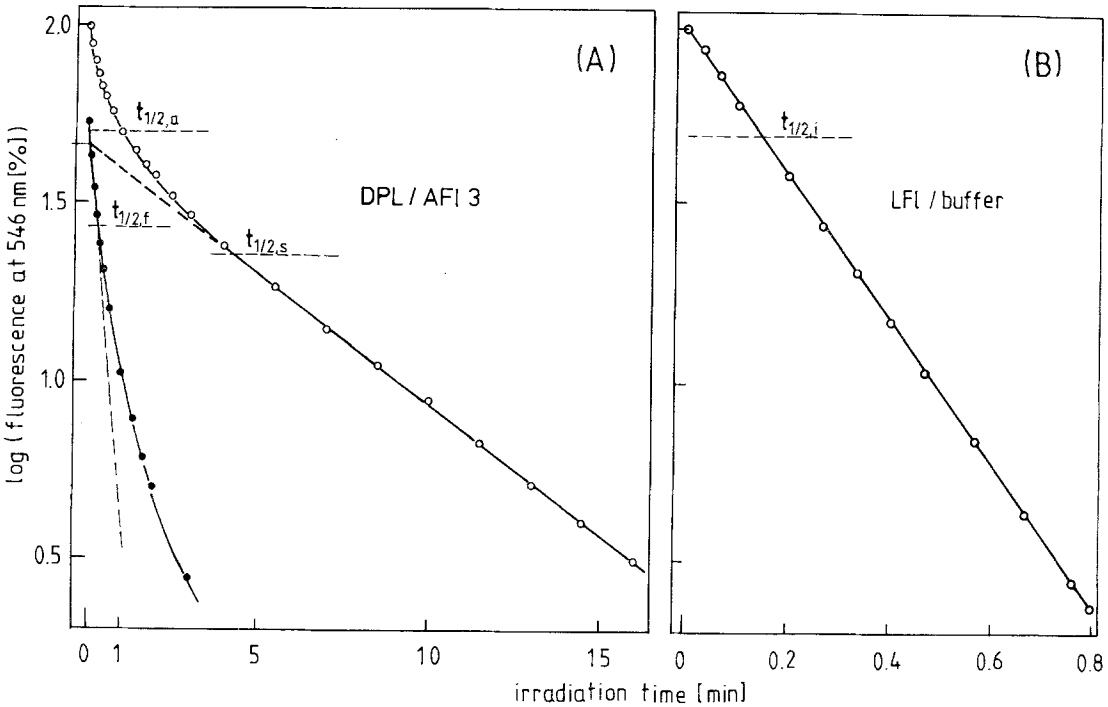

Fig. 9. Half-logarithmic plots of flavin photoreduction kinetics as measured by fluorescence. (*A*): DPL/AFl 3 vesicles at 45 °C, 0.25 M EDTA. The kinetic (○) is a superposition of a "fast" and a "slow" component with an averaged halflife of $t_{1/2,a}=60$ sec. The fast component decays with a halflife of $t_{1/2,f}=15$ sec, the slow one within several minutes ($t_{1/2,s}=5$ min). A concentration ratio of fast/slow ≈ 1:1 is determined by the intercept (dashed lines). (*B*): Aqueous (i.e., isotropically dissolved) lumiflavin exhibits a halflife of $t_{1,2,i}=9$ sec (0.1 M EDTA, 21 °C, pH 8)

Table 2. Photoreduction kinetics obtained for different flavin/EDTA/membrane arrangements, as visualized in Fig. 8[a]

System	[Flavin]	Photoreduction
DPL/AFl 3, 45 °C; 0.25 M EDTA either only inside *or* only outside	4 μM fast: slow= 53:47	Two compounds: fast ($t_{1/2}$=15 sec) slow (min)
DPL/AFl 3, 45 °C; 0.25 M EDTA inside *and* outside	4 μM	First order fast ($t_{1/2}$=15 sec)
DPL/AFl 3, 45 °C no EDTA	4 μM	Nil
3Me-lumiflavin, 20 °C; 0.1 M EDTA, isotropic	4 μM	First order fast ($t_{1/2}$=9 sec)
3Me-lumiflavin, 20 °C or 45 °C, isotropic; +flavin-free DPL-vesicles	4 μM	Nil

[a] Clearly, redoxequivalents are somehow transferred across the membrane.

Table 3. Rate constants k_q of fluorescence-quenching of differently arranged flavins as deduced from the *Stern-Volmer* equation on the basis of data like those shown in Fig. 12

System	T[°C]	Quencher	$10^9 * k_q$
DPL/AFl 10	20		1.5
	45	N_3^-	1.1
3-Me-LFl (Buffer)	20		17.0
	45		26.0
DPL/AFl 10	20		5.1
	45	I^-	4.0
3-Me-LFl (Buffer)	20		22.0
	45		35.0
DPL/AFl 10	21		0.075
	45	Cs^+	0.048
3-Me-LFl (Buffer)	21		0.22
Riboflavin (Buffer)	21		17.0
DPL/AFl 10	21	H^+(pH)	1.8
	45		4.9

first-order kinetics (Table 2). Without any EDTA present, the membrane-bound flavin did not show any measurable photoreduction under these conditions. Similarly, flavin-free, pure lipid vesicles are not capable to serve as photosubstrate for the photoreduction of free 3-Me-lumiflavin (Table 2).

Autoxidation of Vesicle-Bound Flavins

Dihydroflavins undergo highly complex reactions with molecular oxygen, as studied in more detail for the autoxidation of $FMNH_2$ (dihydro-FMN) [6]. However, under certain conditions (0.025 mM $FMNH_2$, 0.125 mM O_2, pH 6.3) the reaction appears to be nearly first order with a halflife of about 40 msec. On the basis of similar concentrations and

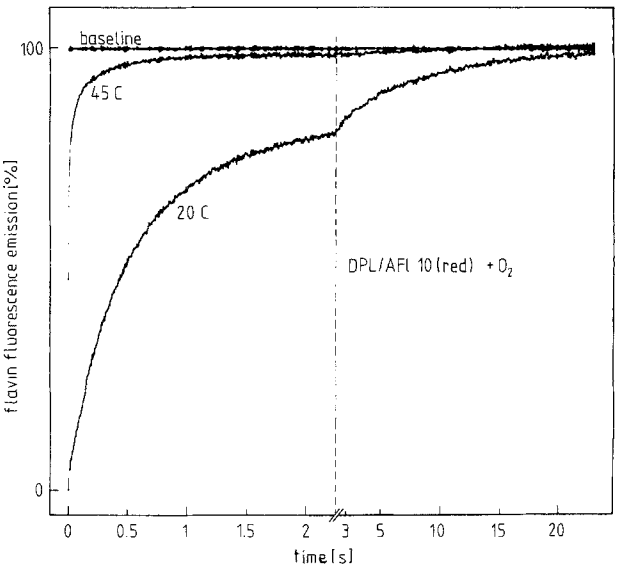

Fig. 10. Reoxidation of nearly fully-reduced (EDTA+light) AFl 10 bound to DPL vesicles by molecular oxygen (air saturated buffer), as measured in a stopped flow apparatus below (20 °C) and above (45 °C) phase transition temperature T_c. Half-logarithmic plots reveal about first-order kinetics, with halflives of 0.5 sec (20 °C) and about 0.05 sec (45 °C). The baseline was taken 0.5 hr after the stopped flow measurements. The kinetics are directly plotted by the on-line computer

pH=8, we also observe apparent first-order kinetics of autoxidation with a halflife of $t_{1/2} \sim 50$ msec for the vesicle-bound flavin, when the membrane is in the *liquid crystalline* state. However, the autoxidation rate is strongly diminished ($t_{1/2}$=0.5 sec), when the membrane is in the *gel* state (Fig. 10). Clearly, the kinetics don't allow us to discriminate between the two distinct flavin localizations (i.e., binding within the inside or outside lipid/water interface; for visualization, *cf.* Fig. 8). The oxygen required for reoxidation was applied by air-saturated buffer ($[O_2] \sim$ 240 μM) in one syringe, and the DPL/AFl 3 suspension in the other syringe of the stopped-flow apparatus.

pH-Dependence of Fluorescence of Membrane-Bound Flavin

The pH-dependency of fluorescence of membrane-bound flavin, in contrast to aqueous riboflavin [16], reveals additional information on membrane properties, as depicted in Fig. 11, again as a function of the membrane phase. First of all, the membrane-bound flavins are highly protected from the outside medium, as reflected by the extended fluorescence quantum efficiency into the acidic and alkaline range. The fluorescence quantum efficiency of AFl 10 bound to DPL vesicles depends on the membrane phase (Fig. 11*A*), however, not so when the flavin chromo-

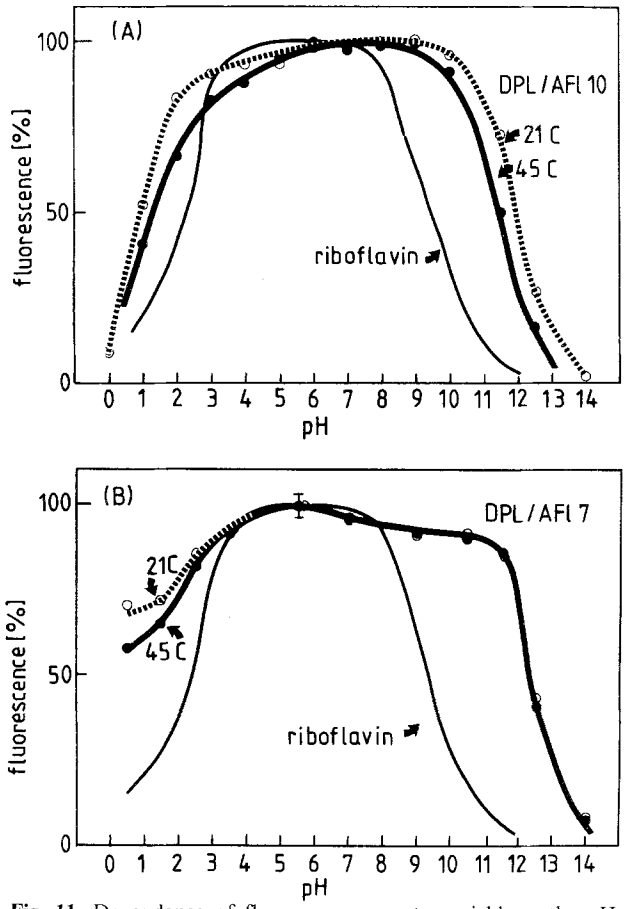

Fig. 11. Dependence of fluorescence quantum yield on the pH. (*A*): DPL/AFl 10. (*B*): DPL/AFl 7. (○) Gel state of the membrane, (●) liquid crystalline state of the membrane, (——) aqueous riboflavin, according to [16]. All curves are normalized to the same height, the pH's are adjusted as described in Materials and Methods. (*Note:* AFl 3 carries the aliphatic chain at N3 and is therefore fully fluorescent up to pH = 13)

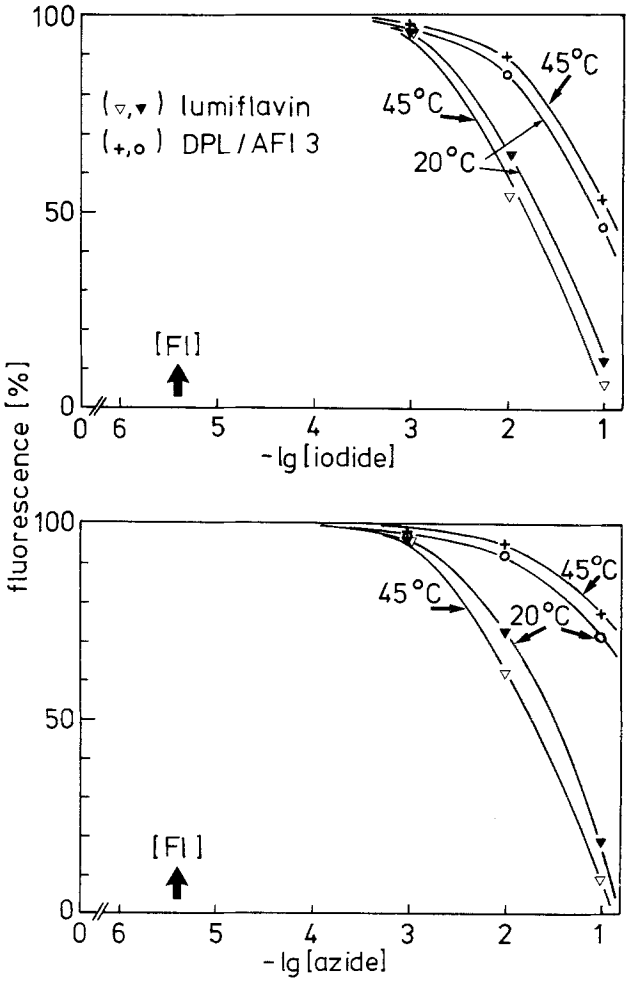

Fig. 12. Relative quantum efficiencies of fluorescence of AFl 10 bound to DPL vesicles, and of aqueous 3-Me-lumiflavin, in the presence of different concentrations of KJ and NaN_3 as fluorescence quenchers. The quenching capability of both quenchers is efficiently decreased for the membrane-bound flavins, being significantly more effective in the *gel* state of the membrane, compared to the *liquid crystalline* state. Under isotropic conditions, the quenchers are more efficient at higher temperatures. The arrows indicate the flavin concentration present (4 μM). This type of measurement has been quantified for several potential quenchers by means of the *Stern Volmer* equation, as listed in Table 3

phore is anchored at position C(7) ("AFl 7") within the membrane (Fig. 11*B*), except for a slight difference below pH 3. The decrease of flavin fluorescence in the acidic range is attributed to proton quenching, in the alkaline range to the pK = 9 of the N(3)-proton (the corresponding anion does not fluoresce [9]).

Fluorescence Quenching of Membrane-Bound Flavins

Quenching experiments reveal further information of the permeability of the interface. As *anionic* quenchers we tested azide and iodide, referring to aqueous 3-Me-lumiflavin (Fig. 12). The essential result is an approximately ten times decreased rate constant of quenching with respect to the membrane-bound flavin, compared to the isotropic case. A smaller but well reproducible and significant effect is the higher effectiveness of these quenchers in the *gel* state of the membrane, compared to the *liquid crystalline* state. As expected (due to the increasing collision rates) in the isotropic case the constants *increase* with increasing temperature, in contrast to the anisotropic case. The same holds true for the cationic quencher Cs^+. The results have been quantified on the basis of the *Stern Volmer* equation (Table 3).

Discussion

The photoreduction kinetics (Fig. 9*A*) and the dependence of the rotational mobility on the viscosity of the bulk solution [23] clearly allow us to discriminate between two populations of membrane-bound flavin with about a 1:1 concentration ratio, in contrast to

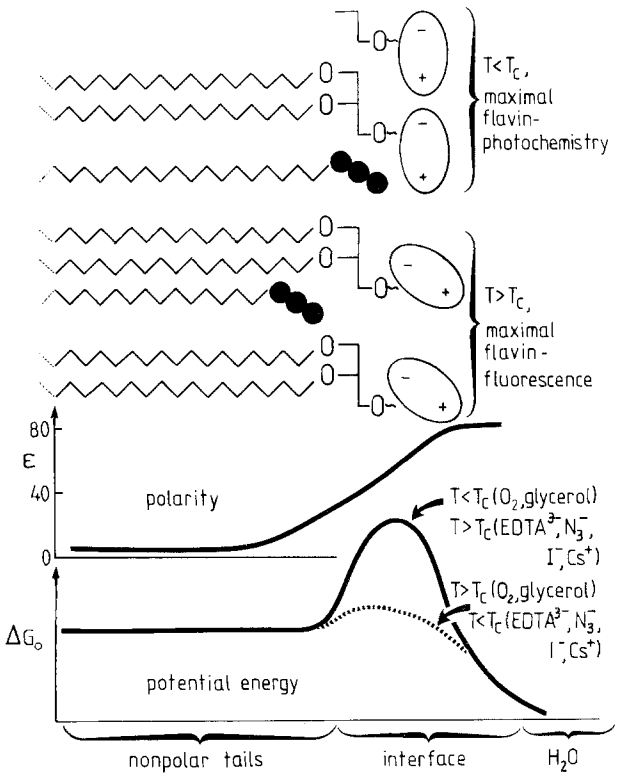

Fig. 13. *Top:* Visualization of the hypothesized phase-dependent localization of membrane-bound flavin (black triple dots), as based on the data qualitatively summarized in Table 4. The polar head groups of the lipids (sketched as ellipses) have been proposed previously to undergo structural changes upon phase transition, as indicated (*cf.* [18, 19]). In the gel state of the membrane the flavin nuclei are mainly confined to the water/lipid interface. Upon the transition from the gel to the liquid crystalline state, they sink into the more hydrophobic part of the membrane. *Bottom:* Qualitative courses of polarity, as seen by the flavin nucleus, and of potential energy, as seen by a variety of flavin "substrates", are depicted as a function of the depth and phase of the membrane. The course of polarity is assumed to be independent of the membrane phase. From the data presented so far, it appears that the penetration barrier for different ions and molecules through the lipid/water interface depends in a highly specific way on the membrane phase, as indicated in the lower part

a single population of *free* flavin (Fig. 9*B*). Control experiments (Fig. 8) demonstrate that the biphasic kinetics are due to the photoreduction of outside and inside, respectively, of the vesicle membrane-bound flavins. Under conditions when *only* external EDTA is the active photosubstrate, redox equivalents have to be transported across the membrane (Figs. 4*A* and 9), since *all* flavins will be reduced in the end. The kinetics (Fig. 9*A*) implies that this trans-membrane transport is the rate-limiting step (for the reduction of the inner-membrane bound flavin). Experiments designed to unravel the mechanism of this transport on the basis of suitable flavin/membrane systems are in progress.

The data of the present and the previous paper [23] strongly support the hypothesis that the membrane-bound flavin nucleus is delocalized from the hydrophilic head-group area of the membrane in the *gel* state, into the more hydrophobic fatty acid region in the *liquid crystalline* state (Fig. 13): (i) its fluorescence quantum yield increases up to 76%, (ii) both its fluorescence emission spectrum and its S2-absorption spectrum are shifted hypsochromically, (iii) the fluidity of its microenvironment increases by a factor of 5 and, concomitantly, its rotational relaxation time decreases by a factor of 5 upon phase transition of the membrane (gel→liquid crystalline). Moreover, (iv) flavin fluorescence quenchers and exogenous photoreduction substrates have easier access to the flavin in the *gel* than in the *liquid crystalline* state, suggesting a smaller barrier in the gel state. (v) Probably the strongest evidence for such a phase-dependent change of microenvironment and delocalization of the flavin is the availability of an internal, membrane-intrinsic electron donor for the flavin triplet in the *gel,* but not in the *liquid crystalline* state, independent of the specific amphiflavin used. The membrane-inherent electron donor gives rise to a quantum efficiency of flavin photoreduction of about $\Phi = 0.02$ (AFl 3, 10; Fig. 4*A, B*). On the basis of comparable concentrations of the "model substrates" octadecan and tributyrin (3 M, Table 1), but under isotropic conditions, the quantum efficiency is ten times smaller (0.002 to 0.004). This highly enhanced speed of photoreduction in the anisotropic case clearly has to be attributed to the stereo-specific mode of flavin/membrane interaction. AFl 7 is by a factor 4 less efficiently photoreduced than AFl 3 and 10 (Fig. 4*C*). High concentrations of EDTA ($> 10^{-2}$ M) have good access to AFl 3 (Fig. 4*A, B*). For chemical reasons we suggest the marked "secondary" hydrogen (Fig. 7) of the glycerol backbone of the lipid to be the electron source, since phosphatidylcholine as one constituent of the membrane/water interface does not act as flavin photosubstrate at all, but tributyrin does under isotropic conditions (Table 1). Although octadecan is capable of acting as an electron donor under isotropic conditions, the aliphatic chains of the membrane lipids obviously do not when they come in close contact with the flavin nuclei above phase transition (similarly as the C_{18}-hydrocarbon chains of the amphiflavins themselves are not or at the most are very poor photosubstrates for the flavin nucleus).

It must be emphasized that the observed maximal decrease of the photoreduction rate mediated by the internal donor (Fig. 6) comes along with the *prephase* of membrane melting, whereas the observed maximum of increase of flavin fluorescence [23] comes along with the *main* phase transition. Thus, the pho-

toreactivity depends on the special conformation of the polar head groups, while the microenvironment of the flavin is made up not by the polar heads but rather by the central glycerol region and, at increased temperature, by the aliphatic chains (*cf.* Fig. 13). This appears to be a logical result which corroborates the assignments of the earlier authors [18, 19].

In contrast to the rate-limiting step of the transmembrane transport of redox equivalents for the photoreduction of the inside of vesicle membrane-bound flavins, transmembrane transport of molecular oxygen is *not* the rate-limiting step for flavin autoxidation as the monophasic kinetics of reoxidation of membrane-bound dehydroflavins suggest (Fig. 10). However, temperature (i.e., membrane phase) has a surprisingly strong influence on the autoxidation rate. In the *liquid crystalline* state the autoxidation rate ($t_{1/2} \sim 50$ msec) is comparable to that observed under isotropic conditions ($t_{1/2} \sim 40$ msec [6]). In the *gel* state, however, it is decreased by a factor of about 10 ($t_{1/2} \sim 500$ msec). This feature, again, reflects properties of the interface: Autoxidation of dihydroflavin is known to be much faster in *hydrophilic* than in *hydrophobic* solvent. Therefore, we would expect a higher autoxidation rate of membrane-bound flavins *below* phase transition, in contrast to the actual observation. This is further consistent (i) with our previous finding [23], that external glycerol molecules have no access to vesicle-bound flavins below T_c, in contrast to the liquid crystalline state, and (ii) with the fact that for the DPL/AFl 10 system protons have easier access to the flavins above rather than below phase transition temperature (Fig. 11*A*).

An additional, highly attractive explanation for the strong inhibition of autoxidation of vesicle-bound flavins ($T < T_c$) is the well-known "salting out" phenomenon [17]. This effect has been originally exploited for the stabilization of oxygen-labile enzymes by charged surfaces of polyionic solid adsorbents. For the case of the vesicle/flavin system we might assume competition between the highly charged vesicle/water interface and oxygen for the solvent, the interface progressively removing water by hydration. This, in turn, would decrease the solvent volume available to oxygen and the autoxidation rate (cf. Fig. 4 in [6]). Due to the different autoxidation rates in the gel and liquid crystalline state, we would – again – expect a dramatic change (of the pattern of charge distribution) of the interface with the membrane phase.

However, a higher permeability of the membrane/water interface above T_c is by no means a general feature, as the fluorescence quenching experiments demonstrate (Fig. 12; Table 3). Both anionic flavin fluorescence quenchers such as azide or iodide, and the cationic quencher Cs^+ are 25 to 35% more efficient *below* than above phase transition temperature. No *biphasic* quenching capability is observed (instead, fluorescence decreases with increasing quencher concentration), suggesting that the interface is the quenching rate-limiting step, rather than the transmembrane transport of the quencher. These and our previous findings [23] are summarized in Table 4 and crudely visualized in Fig. 13. It appears that the energy barrier represented by the interface is higher for neutral molecules (oxygen and glycerol) and lower for ions (EDTA, N_3^-, I^-, Cs^+, H^+) in the gel state of the membrane, and *vice versa*. The polarity, as seen by the flavin within the membrane in the different phases, is deduced from fluorescence quantum efficiencies and spectral peak positions ([23], Fig. 2). Interestingly, for the pH-dependency of fluorescence quantum efficiency of AFl 7 bound to DPL-vesicles no phase dependency (except a very small one in the acidic range [Fig. 11*B*]) could be found, in contrast to AFl 10. This again has to be attributed to the specific membrane/flavin interaction. As originally postulated by Blumenthal et al. [1], the lipid/water interface turns out to be an essential entity which has to be included in any theory on (biological) membrane transport, even if its exact definition in physiological terms cannot yet be given. The phenomena described here and in the previous paper [23] clearly favor its existence.

Table 4. Trends of reactivity of membrane-bound flavins as a function of membrane phase, compared to the isotropic cases

	Low T isotropic	$T < T_c$ anisotropic	High T isotropic	$T > T_c$ anisotropic
Fluorescence	High	Low	Low	High
Photoreduction rate	High	High	Low	Low
Quenching rate	Low	High	High	Low
Autoxidation	Low	Low	High	High
Polarity of microenvironment	–	High	–	Low
Flavin access by external glycerol	–	Nil	–	High

Realistically, all three effects discussed so far (inside/outside problem, phase-dependent delocalization and phase-dependent structure and permeability of the interface) influence the accessibility of membrane-bound flavins simultaneously, but with different efficiency, and cannot be separated from each other in a clear-cut way. Apart from any interpretation, we learned from our studies so far, that (i) membrane-bound flavins are highly separated ("protected") from the bulk medium, (ii) that their reactivity can be controlled by the position of anchoring ("amphi-

flavin") and the specific (photo-)substrate used, and (iii) that the phase of the membrane plays a predominant role (note, in biological membrane systems such *apparent* phase transitions as investigated here are never seen which, however, does not exclude control of biological membrane-bound molecules by "microphase transitions", which remain undetectable by common assays).

The following relation to photo-physiology needs to be mentioned. The flavin-mediated, *in vivo b*-type cytochrome photoreduction as observed in several bluelight-sensitive organisms, is being discussed as a possible sensory transduction step (for details *see* [24]). Lipson and Presti [20] argue against the assumed physiological relevance of this reaction, since it occurs with a quantum efficiency of only about 0.015, far below an expected physiologically relevant photoreaction quantum efficiency near unity. Figures 4 and 5 show that the quantum efficiency of flavin-photoreduction never exceeds 0.05, supporting the artefactual nature of the cytochrome *b* photoreduction *in vivo*. Nevertheless, there is overwhelming evidence accumulating that the physiological bluelight receptor is indeed a flavin-mediated photo-redox-reaction [24]. This, in turn, implies that nature employs a flavin photoreduction ($1e^-$ or $2e^-$?) with a quantum efficiency some 20 times higher than the most sensitive "artificial" one described here (DPL/AFl 10/EDTA or BH_3CN^-, *gel* phase, $\Phi = 0.05$).

Recently a study was published by Heelis et al. [8] entitled "Flavin Pigments Embedded in Liquid Matrices: A Spectroscopic and Photochemical Investigation." Unfortunately, due to questionable interpretations and experimental aspects this paper requires revision: Since Heelis et al. did not purify the sonicated suspension, we assume that most of the flavin they measured is *not* vesicle-bound but rather micellar. In addition, these authors used flavin concentrations at least 10 times higher ([flavin]/[lipid] = 10/100) than the maximum vesicle load possible, as determined independently by Trissl [28] for flat, and Schmidt [23] for vesicular membranes ([flavin]/[lipid] = 1/100). Moreover, they observed flavin fluorescence depolarization with increasing flavin concentration, which was taken to indicate that at "high values of [flavin]/[vesicle] the flavins can no longer be packed into the liposome...". However, some 25 years ago Weber already [30] developed a theory extracting information about the distance and interaction of fluorphores out of this well-known concentration-dependency of fluorescence polarization (in isotropic solution).

Moreover, Heelis et al. did not differentiate between aerobic and anaerobic photoreactions. However, flavin photochemistry done in the presence of oxygen is largely meaningless for two reasons: (i) The flavin triplet may produce singlet oxygen, which, in turn, is known to induce a series of irreversible degradation reactions. (ii) Besides this, oxygen reacts rapidly with the reduced flavin formed photochemically, yielding peroxide which will interfere with the system as a whole [*cf.* 26]. Thus, Heelis et al. are in disagreement with the very bulk of flavin literature if they claim that they observed the same results under aerobic and anaerobic conditions.

Finally, Heelis et al. did not distinguish between the two fundamentally different photoreactions termed "photolysis" and "photoreduction" [7, 12] and probably observed an overlap of these. It is in this way that we interpret their biphasic kinetics. On this basis their interpretation of "two distinct environments for the flavin chromophore" remains highly questionable.

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References

1. Blumenthal, R., Changeux, J.-P., Lefever, R. 1970. Membrane excitability and dissipative instabilities. *J. Membrane Biol.* **2**:351
2. Bruice, R.C. 1976. Models and flavin catalysis. *In:* Progress in Bioanorganic Chemistry. E.T. Kaiser and F.J. Kezdy, editors. Vol. 4, p. 1. John Wiley & Sons, New York – London – Sidney – Toronto
3. Butler, W.L. 1972. Spectroscopy of biological materials. *In:* Methods in Enzymology. Vol. XXIV, Part B, pp. 3–25. A. San Pietro, editor. Academic Press, New York
4. Butler, W.L., Hopkins, D.W. 1970. Higher derivative analysis of complex absorption spectra. *Photochem. Photobiol.* **12**:439
5. Ghisla, S., Hartmann, W., Hemmerich, P., Müller, R. 1973. Die reduktive Alkylierung des Flavinkerns; Struktur und Reaktivität von Dihydroflavinen. *Liebigs Ann. Chem.* **1973**:1388
6. Gibson, Q.H., Hastings, J.W. 1961. The oxidation of reduced flavin mononucleotide by molecular oxygen. *Biochem. J.* **83**:368
7. Haas, W., Hemmerich, P. 1979. Flavin-dependent substrate photo-oxidation as a chemical model of dehydrogenase action. *Biochem. J.* **181**:95
8. Heelis, P.F., Parsons, B.J., Phillips, G.O., Barghigiani, C., Colembetti, G., Lenci, F., McKellar, J.F. 1979. Flavin pigments embedded in lipid matrices: A spectroscopic and photochemical investigation. *Photochem. Photobiol.* **30**:507
9. Hemmerich, P. 1976. The present status of flavin and flavoenzyme chemistry. *Prog. Chem. Org. Natural Prod.* **33**:451
10. Hemmerich, P. 1977. Bio(in)organic views on flavin-dependent one-electron transfer. *In:* Bioorganic Chemistry II. pp. 321–329, Advances in Chemistrys, Series No. 162. K.N. Raymond, editor. Academic Press, New York
11. Hemmerich, P., Ehrenberg, A., Walker, W.H., Erikson, L.E.G., Salach, J., Bader, P., Singer, T.P. 1969. On the structure of succinate dehydrogenase flavocoenzyme. *FEBS Lett.* **3**:37
12. Hemmerich, P., Knappe, W.-R., Kramer, H.E.A., Traber, R. 1980. Distinction of $2e^-$ and $1e^-$ reduction modes of the flavin

chromophore as studied by flash photolysis. *Eur. J. Biochem.* **104:**511
13. Hemmerich, P., Massey, V. 1980. The role of the apoprotein in directing pathways of flavin catalysis. *In:* Oxidases and Related Redox Systems. T.E. King, H.S. Mason, M. Morrision, editors. Pergamon Press, Oxford (*in press*)
14. Hemmerich, P., Wessiak, A. 1979. On the chemistry of flavin-dependent oxygen activation. *In:* Oxygen: Biochemical and Clinical Aspects. W.S. Caughey, editor. pp. 491–511. Academic Press, New York
15. Hinz, H.J., Sturtevant, J.M. 1972. Calorimetric studies of dilute aqueous suspensions of bilayers from synthetic L-α-Lecithin. *J. Biol. Chem.* **247(19):**6071
16. Kavanagh, R., Goodwin, R.H. 1949. The relationship between pH and fluorescence of several organic compounds. *Arch. Biochem.* **20:**315
17. Klibanov, A.M., Kaplan, N.O., Kamen, M.D. 1978. A rationale for stabilizing of oxygen-labile enzymes: Application to a clostridial hydrogenase. *Proc. Natl. Acad. Sci. USA* **75:**3640
18. Ladbrooke, B.D., Chapman, D. 1969. Thermal analysis of lipids, proteins and biological membranes; A review and summary of recent studies. *Chem. Phys. Lipids* **3:**304
19. Lee, A.G. 1975. Functional properties of biological membranes: A physical chemical approach. *Prog. Biophys. Mol. Biol.* **29:**5
20. Lipson, E.D., Presti, D. 1977. Light-induced absorbance changes in *Physomyces* photomutants. *Photochem. Photobiol.* **25:**203
21. Michel, H., Hemmerich, P. 1980. Substitution of the flavin chromophore with lipophilic sidechains: A novel membrane redox label. *J. Membrane Biol.* **60:**143
22. Radda, G.K., Calvin, M. 1964. Chemical and photochemical reductions of flavin nucleotides and analogs. *Biochemistry* **3:**384
23. Schmidt, W. 1979. On the environment and the rotational motion of amphiphilic flavins in artificial membrane vesicles as studied by fluorescence. *J. Membrane Biol.* **47:**1
24. Schmidt, W. 1980. Physiological bluelight reception. *In:* Structure and Bonding. P. Hemmerich, editor. Vol. 41, pp. 1–41. Springer, Berlin–Heidelberg–New York
25. Schmidt, W. 1980. A high-performance dual wavelength spectrophotometer and fluorometer. *J. Biochem. Biophys. Methods* **2:**171
26. Schmidt, W., Butler, W.L. 1976. Flavin-mediated photoreactions in artificial systems: A possible model for the bluelight photoreceptor pigment in living systems. *Photochem. Photobiol.* **24:**71
27. Träuble, H. 1971. Phasenumwandlungen in Lipiden, Mögliche Schaltprozesse in biologischen Membranen. Naturwissenschaften **58:**277
28. Trissl, H.W. 1974. Studies on the incorporation of fluorescent pigments into bilayer membranes. *Biochim. Biophys. Acta* **367:**326
29. Walker, W.H., Hemmerich, P., Massey, V. 1970. Light-induced alkylation and dealkylation of the flavin nucleus. Stable dihydroflavins. Spectral course and mechanism of formation. *Eur. J. Biochem.* **13:**258
30. Weber, G. 1954. Dependence of the polarization of the fluorescence on the concentration. *Trans. Faraday Soc.* **50:**552

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