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ON THE ENVIRONMENT AND THE ROTATIONAL MOTION OF AMPHIPHILIC FLAVINS IN ARTIFICIAL MEMBRANE VESICLES AS STUDIED BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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This paper continues the studies of vesicle-bound flavins ('anisotropic flavin chemistry'). It is possible to anchor the flavin nucleus in various modes within the lipid/water interface by means of long aliphatic chains and using different saturated lipids, thereby mimicking the specific binding of the coenzyme to the apoprotein in flavoproteins. Based on absorption spectroscopy and EPR spectroscopy studies we explored the rotational mobility and the microenvironment of membrane-bound amphiflavin radicals. N(5)-unsubstituted amphiflavin radicals exhibit a similarly high disproportionation constant as known from isotropic flavin chemistry. However, reasonable stabilization of the radical was achieved by introduction of an alkyl group in position 5 in the reduced state prior to the one-electron oxidation. Adopting the fine structure of the corresponding EPR spectra as assay for the mobility of the semiquinone, we determined rotational relaxation times ranging from 60 ns in the crystalline state down to 10 or 15 ns in the liquid-crystalline state of the membrane. The solvatochromic effect shown by absorption spectra of the membrane-bound flavin radicals reflects a dielectric constant of the microenvironment of $\epsilon = 30–40$, corresponding to the lipid/water interface region. The results obtained in this study are consistent with those obtained previously, from fluorescence analyses, supporting our former conclusions.

1. Introduction

Flavin coenzymes are ubiquitous and catalyze a great variety of redox reactions in biochemistry. Three main activities have to be distinguished: (i) (De)hydrogenation, (ii) O_2 activation, (iii) single-electron transfer. Based on this distinction, five main classes of flavoproteins can be formulated [1]. Namely, (1) transhydrogenases, showing activity i only [2], (2) dehydrogenases/oxidases [3] and (3) dehydrogenases/oxygenases, combining activities i and ii [4,5], (4) dehydrogenases/one-electron transferases, combining activities i and iii [2], and finally, (5) flavodoxins, showing activity iii only [6].

The ubiquitous occurrence of flavins in all biological redox chains can thus be explained by the fact that in every such chain there is a critical site at which one- and two-electron equivalents are interconverted. This transformation, especially the splitting of carbanionic substrates into two single electrons and carbonium residues, is unique for flavocoenzymes [7] and differs from any other one/two-electron transformation occurring with ubiquinones [8].

Hemmerich and Massey [9] have recently demonstrated that the type of activity shown by a given flavoprotein is regulated by the apoprotein conformation through regiospecific blocking of the lone pair in either position $1/2\alpha$ or position 5 of the flavin nucleus. The blocking might be achieved either by acidic hydrogen bonds or by positive charge influence from the apoprotein or by both. The flavin chromophore as an azaquinoid system reacts upon $1/2\alpha$ blocking by favored two-electron activity, and upon-position 5 blocking by

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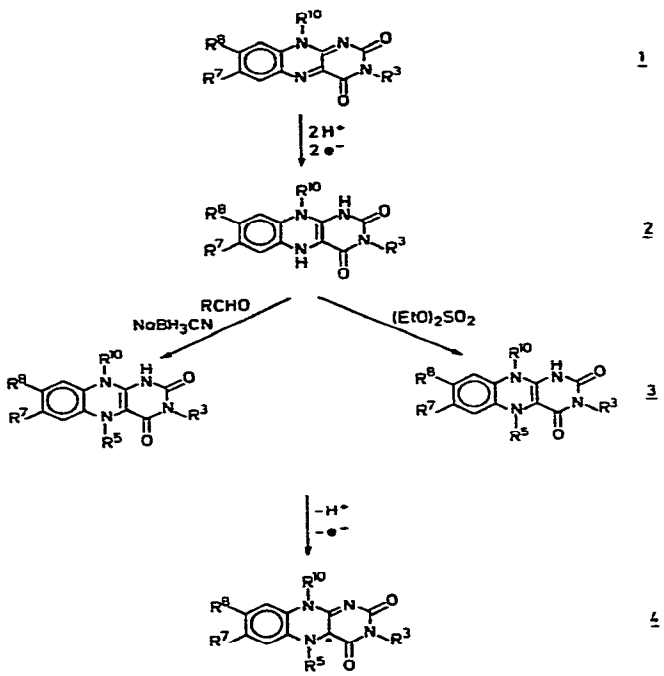
favored one-electron activity. The preference of two-electron activity goes by definition along with destabilization of the radical state, while preference of one-electron activity implies a stabilization of the semiquinone [6]. The biologically essential radical state is the neutral blue semiquinone with maximal absorption in the range 565–640 nm, depending strongly on the polarity of the environment.

In the present study we compare vesicle-bound flavins, localized in a lipid/water interface with those in homogeneous polar or nonpolar solution. By analysis of the radical stabilization we set out from the hypothesis that rotation or shift of the flavin chromophore in an anisotropic environment, such as a membrane/water interface, can mimic conformational changes of flavoapoproteins. Here we study the following properties of the flavin under these conditions: thermodynamic radical stabilization, microenvironment and mobility.

2. Materials and methods

2.1. Chemicals and syntheses (scheme 1)

Solvents and reagents were commercial products of the best available purity. The nitroxide radical spirocyclohexylporphyrexide was synthesized by Dr. G. Blankenhorn [10]. 10-Methyl-3-octadecylisoalloxazine (1a), 7-octadecyl-3,8,10-trimethylisoalloxazine (1c), 3-methyl-10-octadecylisoalloxazine (1d), 10-octadecyl-3,7,8-trimethylisoalloxazine (1g) and 3,10-dimethyl-5-octadecyl-1,5-dihydroisoalloxazine (3b) were synthesized as described earlier [11]. Starting from 3,10-dimethylisoallaxazine (1e) [12] and 3-methyl-10-propylisoalloxazine (1e) [13], we prepared 3,10-dimethyl-5-ethyl-1,5-dihydroisoalloxazine (3e) and 3-methyl-1-propyl-5-ethyl-1,5-dihydroisoalloxazine (3f) according to Ghisla et al. [14]. Reductive alkylation in position 5 was performed under anaerobic conditions according to Müller et al. [15] with the following modifications: A 10^{-3} M solution of the oxidized isoalloxazine in chloroform (3:1, v/v) was shaken with an equal volume



Scheme 1. Preparation of 5-alkylated flavosemiquinones. (a) $R^3 = C_{18}H_{37}$, $R^5 = C_2H_5$, $R^7 = R^8 = H$, $R^{10} = CH_3$ [Afl-3(7,8-H)]; (b) $R^3 = CH_3$, $R^5 = C_{18}H_{37}$, $R^7 = R^8 = H$, $R^{10} = CH_3$ [Afl-5(7,8-H)]; (c) $R^3 = CH_3$, $R^5 = C_2H_5$, $R^7 = C_{18}H_{37}$, $R^8 = R^{10} = CH_3$ [Afl-7(8-Me)]; (d) $R^3 = CH_3$, $R^5 = C_2H_5$, $R^7 = R^8 = H$, $R^{10} = CH_3$ [Afl-10(7,8-H)]; (e) $R^3 = CH_3$, $R^5 = C_2H_5$, $R^7 = R^8 = H$, $R^{10} = CH_3$; (f) $R^3 = CH_3$, $R^5 C_2H_5$, $R^7 = R^8 = H$, $R^{10} = C_3H_7$; (g) $R^3 = CH_3$, $R^5 = C_2H_5$, $R^7 = R^8 = CH_3$, $R^{10} = C_{18}H_{37}$ [Afl-10(7,8-CH_3)]. The reaction path on the right-hand side is valid for the semiquinones 4a, 4d–4g, whereas 4b and 4c were prepared according to the left-hand pathway.

of 0.1 M Na_2HPO_4, which was half-saturated with NaCl for better phase separation, and which contained a 10-fold excess of sodium dithionite. A 20-fold excess of diethyl sulfate was added to the reduced isoalloxazine followed by addition of excess ethyldiisopropylamine after 30 min. The reaction mixture was stirred in the dark at room temperature for 18 h, taking care that the separated phases were not disturbed. After washing with 0.1 M imidazole and 0.1 M acetic acid, the organic phase was concentrated to a small volume

and chromatographed on silica gel (Merck silica gel 60, 70-230 mesh ASTM) with acetic acid ethyl ester under anaerobic conditions. Isoalloxazine 1c was reductively alkylated according to the method described by Kemal et al. [16] with the following modifications: The isoalloxazine solution was reduced as described above and then anaerobically transferred to an argon-flushed solution containing a 20-fold excess of acetaldehyde in acetonitrile (3 M). After 30 min, 2 ml acetic acid and a 100-fold excess of sodium cyanoborohydride were added and then stirred for 8 h at room temperature in the dark. Purification was achieved as described above. The concentrations of the reduced stock solutions in chloroform, stored under reducing conditions (0.1 M Na_2HPO_4, $Na_2S_2O_4$, half-saturated with NaCl) in the refrigerator, were determined by titration with an ethanolic spirocyclohexylporphyrexide solution in the presence of 5% triethylamine.

2.2. Preparation of vesicles

The phospholipids L-β,γ-dimyristoyl-α-phosphatidylcholine (DMPC) (No. 41803), L-β,γ-dipalmitoyl-α-phosphatidylcholine (DPPC) (No. 42556) and L-β,γ-distearoyl-α-phosphatidylcholine (DSPC) (No. 43698) were purchased from Fluka, Switzerland, as analytical grade. A mixture of $5-6 \times 10^{-5}$ mol of the phospholipid dissolved in ethanol and $5-6 \times 10^{-7}$ mol of the 5-alkyl-1,5-dihydroisoalloxazine species was evaporated to dryness under vacuum at 0.67 Pa. The dry film was suspended in 6 ml of 0.01 M Tris ($\mu = 0.1$ M, NaCl), pH 7.8 ± 0.05, and dispersed ultrasonically for 45 min using a sonification bath [17] (Bandelin Sonorex RK 102/B) with an energy supply of 120 W at 35 kHz. The temperature was maintained at 55°C for DMPC and DPPC, and at 65°C for DSPC vesicles. Reduced 5-alkylisoalloxazine species are auto-oxidizable yielding a 4a-hydroxy-5-alkylisoalloxazine [18]. Therefore, we sonicated the dispersions in thin-walled test-tubes (15 mm o.d.) closed with a serum cap, and purged them carefully with argon.

Oxidation to the blue-green membrane-bound isoalloxazine radicals 4a–4d and 4g was achieved by adding a stoichiometric amount of the nitroxide radical [10]. Isoalloxazine radical concentrations were determined spectrophotometrically using extinction coefficients which were obtained from the titrations of the reduced stock solutions, i.e., 4a 620(3900); 4b, 618(3900); 4c, 630(4300); 4d, 620(3900); 4g, 630(4300) [nm (M^{-1} cm^{-1})]. Unfortunately, due to the oxygen sensitivity of the membrane-bound isoalloxazine radical, a further chromatographic purification of the sonicated lipid suspension is not feasible under normal conditions. However, column chromatography (Sepharose 4B) of lipid suspensions yielded about 80% vesicles (based on total lipid inserted) with a diameter of 200–250 Å, as judged by the elution profile. This reasonable efficiency was taken as justification for leaving out this purification step. Vesicles, charged with oxidized amphiflavins, were prepared and purified as described by Schmidt [19].

2.3. Spectroscopy

Absorption spectra of vesicle-bound isoalloxazine radicals and oxidized isoalloxazine were measured in 1 cm cuvettes with a Cary Superscan 3 spectrophotometer, using as the scattering reference flavin-free vesicles prepared in the same manner as the flavin-loaded vesicles.

EPR spectra were recorded on a Varian E 109 ES spectrometer with 100 kHz field modulation and nonsaturating conditions, using flat quartz cells. The temperature was controlled with a Varian V-4257 variable temperature accessory and measured with a chromel-alumel thermocouple using a Newport Instruments digital thermometer model 268 (accuracy ± 0.5°C). For all experiments oxygen was removed either by purging with purified argon or by degassing the samples on a vacuum-line setup. EPR spectra were manually digitized (14 points/mT) with a Hewlet-Packard digitizer (model 9864 A), smoothed with a desk calculator (model 9820 A) and replotted using a plotter (model 9862 A).

3. Results and discussion

3.1. Disproportionation of N(5)-unsubstituted flavin radicals in anisotropic environment

Upon half-reduction of degassed AFl-10(7,8-H) in DPPC vesicles with blue light (450 nm) in the

presence of a 500-fold excess of EDTA [20], only a small amount of the neutral blue semiquinone was detected by its absorption at 580 nm. Based on an extinction coefficient of about 4000 M^{-1} cm^{-1} at 580 nm, we estimate a semiquinone yield of 1.5% per total flavin concentration. This indicates that the membrane-bound blue semiquinone is disproportionated similarly to that dissolved isotropically [21].

Starting from a vesicle dispersion containing 5×10^{-5} M amphiflavin, we would expect a total semiquinone concentration of about 7.5×10^{-7} M. However, EPR spectroscopy requires an at least 10-times higher radical concentration [22]. In order to achieve a sufficient flavin semiquinone concentration, we used N(5)-alkylated flavin radicals, which do not disproportionate [15].

3.2. Absorption spectroscopy

The long-wavelength absorption band can be taken as a sensitive indicator for the environment of isoalloxazine radicals (4e) (for structures, cf. scheme 1). In aqueous systems, we observed a peak at 567 nm, and a shoulder at 605 nm. Ethanol as a polar protic solvent shifts these peak positions bathochromically to 585 and 612 nm, the peaks being now clearly separated. In dipolar aprotic solvents (acetonitrile), these two maxima show up at 586 and 621 nm and in nonpolar solvents (benzene) at 600 and 648 nm (cf. fig. 1). Substitution of the hydrogen atoms in positions 7 and 8 by methyl groups results in a further bathochromic shift of 10–15 nm of these two peaks [23] (cf. fig. 1).

Consistently, vesicle-bound isoalloxazine radicals show a broad absorption band between 590 and 620 nm for the 7,8-unmethylated species (4a and 4b). AFl-7(8-Me, 5-Et) (4c) and AFl-10(7,8-Me, 5-Et) (4g), both carrying methyl groups in positions 7 and 8, absorb between 600 and 630 nm (fig. 2). This absorption range, characteristic for dipolar aprotic solvents, indicates that the membrane-bound flavin radicals are embedded in the polar head group region ('interface') of the membrane with a dielectric constant between 30 and 40 [32]. The hydrophobic part of the membrane, the lipid chains, exhibits a rather low dielectric constant ($\epsilon \approx 2$–4) [22], and the resulting absorption spectra should be similar to those taken in benzene. From this we conclude that the microen-

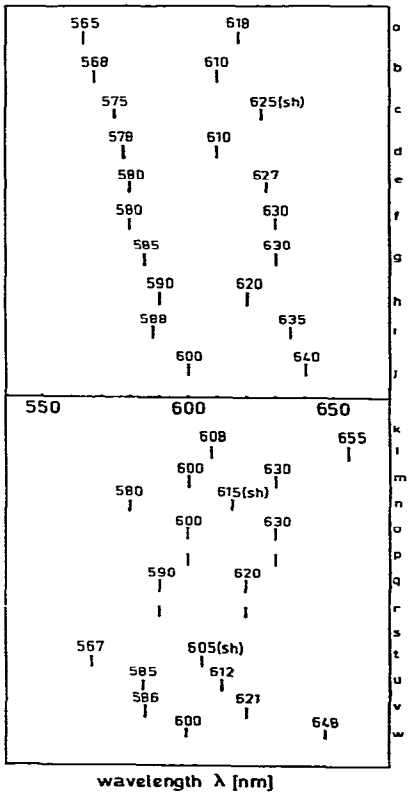


Fig. 1. Peak positions of the long-wavelength absorption band of flavoproteins forming the blue semiquinone. Lower part: Peak positions of N(5)-alkylated flavin radicals in aqueous, dipolar aprotic (acetonitrile), and nonpolar (chloroform) solvents. The peak positions of membrane-bound flavin radicals 4a–4c and 4g are also indicated. Upper: [a] acyl-CoA dehydrogenase [24], [b] bacterial luciferase [25], [c] glucose oxidase [26], [d] *Azotobacter vinelandii* flavodoxin [6], [e] *Peptostreptococcus elsedenii* flavodoxin [27], [f] adrenodoxin reductase [28], [g] NADPH-cytochrome *P*-450 reductase [29], [h] D-amino-acid oxidase [23], [i] ferredoxin-NADPH reductase [30], [j] ferredoxin-NADPH reductase [31]; Lower: [k] N(5)-ethyllumiflavin semiquinone [23], [l] benzene, [m] ethanol, [n] buffer, [o] AFl-7(5-Et, 8-Me), in DPPC vesicles, [p] AFl-10(5-Et, 7,8-Me), in DPPC vesicles, [q] AFl-3(5-Et, 7,8-H), in DPPC vesicles, [r] AFl-5(7,8-H), in DPPC vesicles, [s] N(5)-ethylisoalloxazine semiquinone 4e, [t] buffer, [u] ethanol, [v] acetonitrile, [w] benzene.

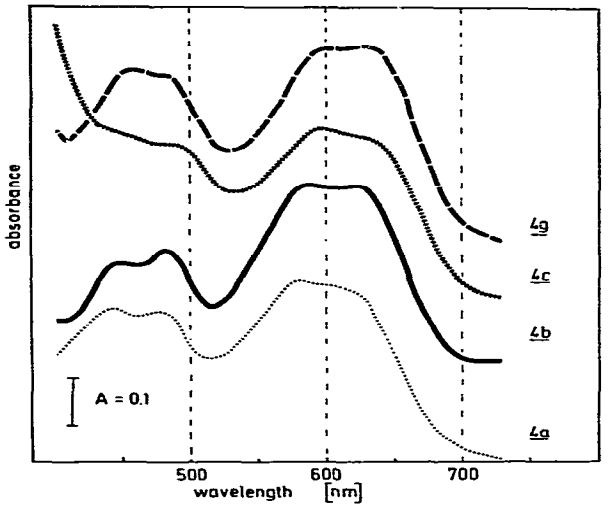


Fig. 2. Visible spectra of flavin radicals 4a–4c and 4g, bound to DPPC vesicles. The absorption are normalized to a radical concentration of 10^{-4} M. The spectra were recorded at 30°C.

vironment of membrane-bound isoalloxazine radicals is dipolar. This is in good agreement with our previous conclusions based on spectroscopic analyses of the oxidized flavin/vesicle system [19].

3.3. EPR spectroscopy of free 5-ethylated isoalloxazine radicals

Isoalloxazine radicals 4e and 4f in aqueous solutions exhibit well resolved EPR spectra (fig. 3). The EPR spectrum of 4e consists of 25 lines with a mean distance of 0.2 mT. The total width of this radical spectrum is 4.91 mT. Substitution of the 10-methyl group by a propyl group will influence the spectrum: as many as 21 lines with a mean spacing of 0.2 mT are resolved and the total width is reduced to 4.2 mT. The difference of the total widths can be attributed to the restricted rotation of the methylene group in 4f compared to the free-rotating methyl group in radical 4e. From the known hyperfine coupling constant $a_H(10\text{-}CH_3) = 0.39$ mT [15], we calculate a coupling constant $a_H(10\text{-}CH_2) = 0.21$ mT for the methylene hydrogens in radical 4f. It was shown by Maruani [33,34] that the conformational dependence of hyperfine coupling is described by a Fourier series of the angle ϕ between the nitrogen π-orbital and the plane containing the β-proton C-H bond. In the present case of nearly identical geminal substituents at N(10), this series can be essentially reduced [35]:

$$a_H = B_0 + B_2 \cos^2\phi. \quad (1)$$

As a first approach, the correction term B_0 was neglected. This term was added for spin-density transmission by spin polarization [36]. Therefore,

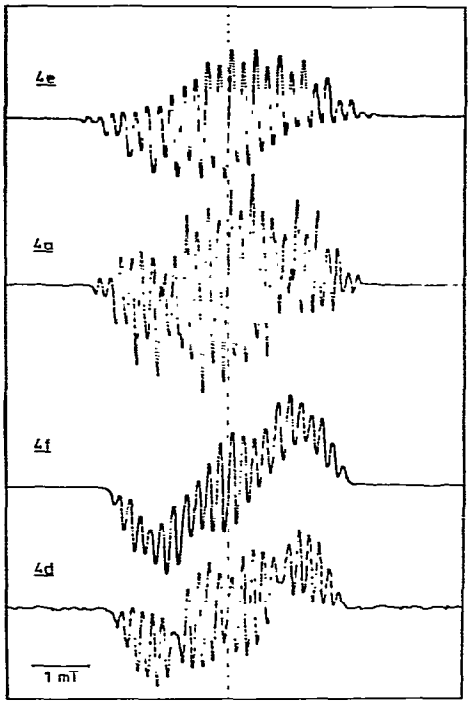


Fig. 3. EPR spectra of flavin radicals 4e and 4f and amphiflavin radicals 4a and 4d at high mobility (isotropic spectra). 4e and 4f were dissolved in buffer of pH 8.2, whereas the semiquinones with lipophilic side chains in position 3 or 10, respectively, were dissolved in ethanol. Substitution of the methyl by an octadecyl group in position 3 has no influence on the total width and number of lines in the EPR spectra (cf. 4a and 4e). Note the influence of the substituent in position 10. A propyl or an octadecyl group reduces the total width and yields a smaller number of lines.

we calculate from the data of the free-rotating methyl group ($\langle\cos^2\phi\rangle = 0.5$) B_2 to be 0.78 mT. On this basis, we obtain for the angle between the π-orbital and the β-proton C-H bond a value of approx. 60°, in good agreement with investigations on 10-substituted phenothiazine cation radicals [36]. Fig. 3 also shows the EPR spectra of 5-ethylated amphiflavin radicals 4a and 4d in ethanolic solution. These spectra are similar to those of the corresponding semiquinones 4e and 4f in aqueous solution. An octadecyl substituent in position 3 does not alter the EPR spectrum compared to that of the N(3)-methyl derivative. Therefore, the spin density in this position can be neglected [15]. On the other hand, position 10 is known to exhibit some spin density [15]. However, the EPR spectra of both 10-octadecyl (4d) and 10-propyl (4f) derivatives compare well (fig. 3). This justifies the adoption of the isotropic EPR spectra of semiquinone 4f as a calibration standard for the rotational relaxation time of the vesicle-bound radical 4d.

3.4. EPR spectroscopy of membrane-bound isoalloxazine radicals

EPR spectra of 5-alkylated isoalloxazine radicals (4a–4d) bound to phospholipid vesicles are shown in figs. 4 and 5. The spectra do not show hyperfine splitting at moderate temperatures, reflecting significant demobilization. The line-broadening effect can be mainly attributed to anisotropic electron-spin nuclear-spin interactions. Broadening mechanisms such as dipole-dipole or electron-electron exchange interactions, which are of intermolecular origin, can be largely excluded at the low radical concentration used [22]. The molar ratio of lipid to flavin radical never exceeded 0.01, equivalent to an intermolecular distance of at least 70 Å [22].

3.5. Anisotropic effects and rotational correlation times

The difference between the axial components of the T tensor of the two nitrogen atoms in positions 5 and 10 and the corresponding isotropic coupling constants of 0.8 and 0.36 mT, [15], respectively,

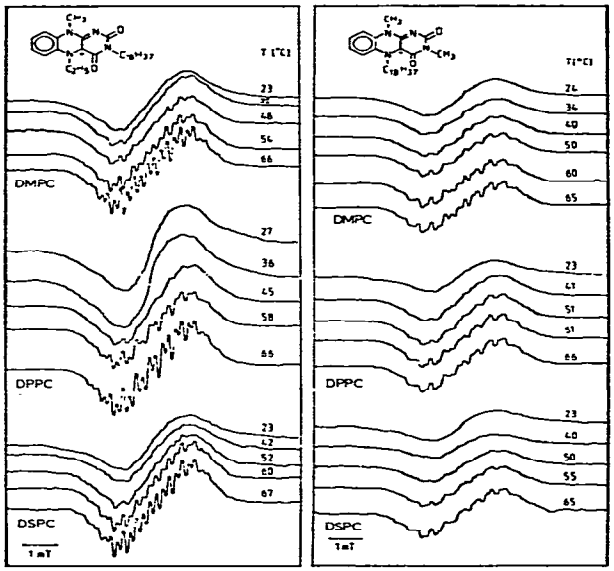


Fig. 4. EPR spectra of membrane-bound 5-alkylated flavin radicals as a function of temperature (membrane phase). Left: AFl-3(7,8-H, 5-Et) (4a) in DMPC-, DPPC and DSPC vesicles; Right: AFl-5(7,8-H) (4b) in DMPC, DPPC and DSPC vesicles.

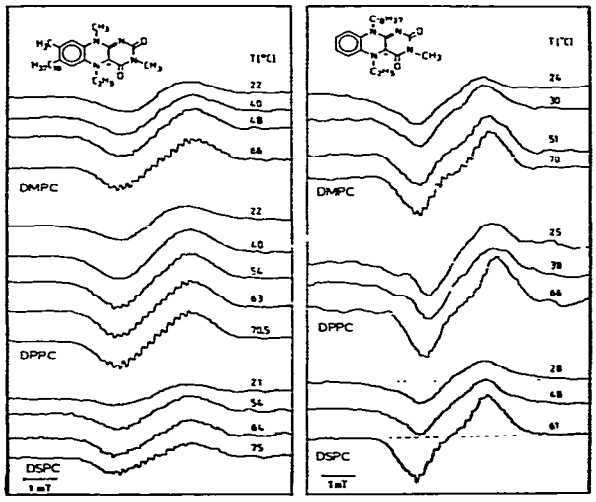


Fig. 5. EPR spectra of membrane-bound 5-alkylated flavin radicals as a function of temperature (membrane phase). Left: AFl-7(8-Me, 5-Et) (4c) in DMPC, DPPC and DSPC vesicles; Right: AFl-10(7,8-H, 5-Et) (4d) in DMPC, DPPC and DSPC vesicles.

can be regarded as a measure of the anisotropy [37]. Assuming axial symmetry, $T_{\parallel}$ has a value of $2.5 \times a_N$, and $T_{\perp}$ was proposed to be $0.25 \times a_N$. The anisotropic hyperfine interaction is then expressed by the following formula:

$$\Delta\omega = \sum (T' - a'_N) \quad (2)$$

and is of the order of 30 MHz, after conversion to angular frequency units. To average out all anisotropic hyperfine interactions, the rotational correlation time τ_c must be small compared with $\Delta\omega^{-1}$ [38], which is the case for the free isoalloxazine radicals 4e and 4f exhibiting well resolved EPR spectra (fig. 3).

EPR spectra were recorded for isoalloxazine radicals anchored via the lipid chain in the 3-, 5-, 7- and 10-positions within the specific phospholipid vesicles. The temperature was varied between 22 and 70°C to cover the region of the lipid phase transitions [39]. At temperatures below that of the specific phase transition (crystalline state), the incorporated radicals are largely immobile as indicated by the broad, unresolved EPR spectra. The radicals are statistically distributed in the vesicles, i.e., their magnetic moments interact isotropically with the applied magnetic field, thus leading to the unresolved powder-like EPR spectra. The spectral widths reflect the anisotropy of the nitrogen hyperfine tensors. As shown in figs. 4 and 5, raising the temperature above the corresponding transitions temperatures T_c (liquid-crystalline state) results in a decreased total width of the spectra and in some cases splitting appears. At these temperatures, the membrane has changed its physical state and the lipid chains become more fluid due to conformational changes of the aliphatic chains. Moderately resolved EPR spectra of the incorporated radicals reflect this greater fluidity, especially in the case of isoalloxazine radical 4a. A less pronounced splitting is exhibited by the isoalloxazine radicals 4b and 4c, anchored via the 5- or 7-position within the membrane. However, splitting is scarcely seen even at elevated temperatures for the isoalloxazine radical 4d incorporated into the membrane via the 10-position. These different temperature dependences can be explained by differently facilitated reorientations due to their specific incorporation into the phospholipid membranes. A long alkyl chain in position 3 can be aligned nearly parallel to the long molecular axis of the radical 4a. Reorientation movements around this axis happen easily without any larger displacements of the neighboring lipid chains, and therefore the (hyper)fine structure of EPR spectra (fig. 4) occurs upon temperature increase. A similar characteristic is observed for the isoalloxazine radicals 4b and 4c, attached via positions 5 and 7, respectively. The anomaly of radical 4d can be rationalized as follows:

In this compound, the lipophilic side chain is aligned perpendicular to the long axis, which should be located essentially parallel to the membrane surface. From this assumed perpendicular arrangement of the two axes, a rotation around the long molecular axis appears to be less favorable. On the other hand, rotation around the N(5)–N(10) axis results in a large distortion of the microenvironment, which inhibits easy reorientations as required to obscure the anisotropic interactions. Therefore, no (or very little) hyperfine splitting is obtained even at temperatures far above that of the phase transition ($T > T_c$). Schindler and Seelig [40] observed two different rotational correlation times for a steroid nitroxide spin label (a rod-like molecule), which is incorporated in membranes: Rotation around the long molecular axis is one order of magnitude faster than rotation perpendicular to it.

3.6. Determination of rotational relaxation times

Fig. 6 demonstrates a clear correlation between the rotational correlation time τ_c and the hyperfine splitting. On this basis, the rotational relaxation time ρ of vesicle-bound flavin radicals was determined by comparison of their EPR spectra with those obtained from isotropically dissolved flavin radicals. In order to exclude intermolecular interactions (see above), a low concentration ($\approx 10^{-4}$ M) was chosen.

First, the rotational correlation time τ_c is determined by the law of Stokes-Einstein

$$\tau_c = 4\pi r^3 \eta / (3kT) \quad (3)$$

where $r = 5.2$ Å (effective radius of the radical

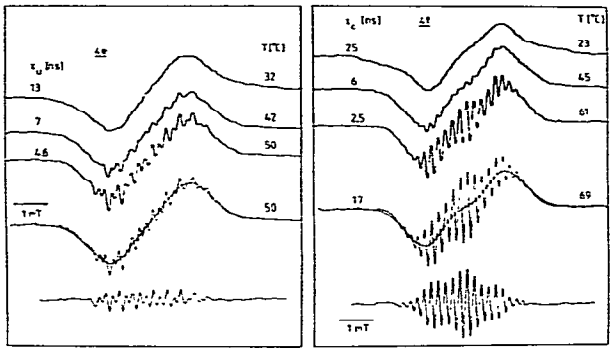


Fig. 6. EPR spectra of 5-ethylisoalloxazine radicals 4e and 4f in glycerol/water (9:1, v/v) as a function of temperature (i.e., viscosity). Rotational correlation times τ_c are included (for determination see text). Bottom: Examples of difference spectra Δ (original spectrum − smoothed spectrum) taken at 50°C (left) and 69°C (right), which was used to assay the rotational relaxation time of vesicle-bound flavin radicals (for details see text).

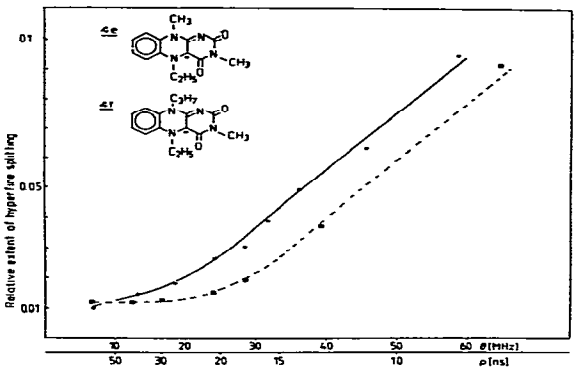


Fig. 7. The relative extent of hyperfine splitting, a measure for the mobility, as a function of the calculated rotational relaxation times ρ for the two 5-ethylisoalloxazine radicals 4e (●) and 4f (■). The x-axis also contains the corresponding values of the rotational diffusion constant θ, which were obtained from the formula below. Note: At rotation relaxation times longer than 25 ns the relative extent of hyperfine splitting reaches a constant level. The semiquinones are largely immobile, exhibiting no resolved EPR spectra. $\theta = kT/8\pi R^3\eta$; $\rho = 4\pi R^3\eta/kT$; $\tau_c = 4\pi R^3\eta/kT$.

[19]), k is Boltzmann's constant, T the absolute temperature, and η the solvent viscosity, determined by the formula

$$\ln \eta = A + B/T \qquad (4)$$

with $A = -6.326$ and $B = 2546\ \mathrm{K}^{-1}$ [41].

Second, the hyperfine splitting of the EPR spectra was quantified as follows (cf. fig. 6: 50°C, left; 69°C, right): The EPR spectrum was smoothed until it was void of hyperfine splitting. The difference spectrum Δ (original − smoothed) was calculated (fig. 6, bottom) and succeedingly squared. Finally, the squares of all points composing the spectrum (digitized by 120 points per spectrum, i.e., 14 points/mT) were added and divided (normalized) by the absolute peak height of the smoothed EPR spectrum. This final value ('relative extent of hyperfine splitting') was determined for the two isoalloxazine radicals 4e and 4f as a function of the rotational relaxation time ρ and taken as calibration standard (fig. 7).

The EPR spectra of the flavin radicals bound to phospholipid vesicles were treated similarly. The resulting rotational relaxation times, which depend on the temperature and also on the phospholipids employed, are shown in fig. 8. At high temperature ($T > T_c$), the rotational relaxation times reach a value of 10–12 ns, whereas at temperatures below the specific phase transition we estimate 40–60 ns. These values are in good agreement with those

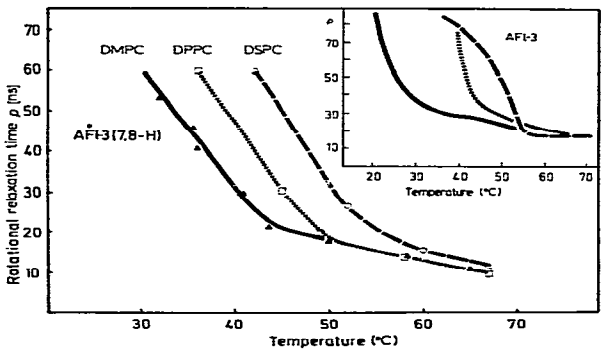


Fig. 8. Rotational relaxation times of the vesicle-bound amphiflavin radical 4a [AFl-3(7,8-H, 5-Et)] as a function of temperature. The corresponding phase-transition temperatures are 23°C for DMPC, 41°C for DPPC and 58°C for DSPC vesicles [37]. Inset: Rotational relaxation times of the oxidized system [Afl-3(7,8-Me)] as obtained from fluorescence analysis [19].

obtained from oxidized amphiflavins by fluorescence polarization measurements (cf. inset, fig. 8) [19].

3.7. Biological aspects

The absorption spectra of various flavoprotein radicals — their peak positions for the long-wavelength absorption bands are shown in fig. 1 — are very different due to the specific influence of the protein environment. The two peak positions range from 568 and 610 nm for bacterial luciferase [25] up to 600 and 640 nm for ferredoxin-NADPH reductase [31]. The pair of lower wavelength values accounts for an aqueous environment, whereas a bathochromic shift of the second pair reflects a nonpolar hydrophobic environment and a restricted accessibility of water to the cofactor.

The bulk protein is also responsible for the immobility of the semiquinone. For a fully deuterated flavodoxin radical from *Synechococcus lividus*, a blue-green alga, Norris and Crespi [42] determined the rotational correlation time to 8.1 ± 1 ns by means of the separation of the outer wings on microwave power-saturated EPR spectra. This value corresponds to a rotational relaxation time of about 24.3 ns. Our results show that flavin radicals, localized in a lipid/water interface, can serve as a biological model describing the environment and mobility of the flavin cofactor in flavoproteins.

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