Anion Formation of 4′-(Dimethylamino)-3-hydroxyflavone in Phosphatidylglycerol Vesicles Induced by HEPES Buffer: A Steady-State and Time-Resolved Fluorescence Investigation

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3-Hydroxyflavones are characterized by an excited-state proton transfer reaction between two tautomeric excited states, which results in two emission bands well separated on the wavelength scale. Due to the high sensitivity of the relative intensities of the two emission bands to solvent polarity, hydrogen bonding, and the local electrical field, these dyes found numerous applications in biomembrane studies. In the present work, we evidenced a new band strongly shifted to the red in both absorption and excitation spectra for 4′-(dimethylamino)-3-hydroxyflavone (probe F) in the presence of anionic phosphatidylglycerol vesicles. Excitation of this new band provided a single-band emission spectrum with a maximum around 530 nm that differs from the two-band profile obtained at lower excitation wavelengths. Furthermore, an additional long-lived lifetime (3.8 ns) was observed in the time-resolved decay only when the probe was excited in the wavelength range of the new excitation band. These spectroscopic features were observed at neutral pH in HEPES but not phosphate−citrate−borate buffer. From their pH dependence, these features could be unambiguously attributed to the anionic form of probe F bound at the interface of the lipid bilayer. This anion may form even at neutral pH due to the HEPES molecules that bind at the bilayer interface to the negatively charged polar heads of phosphatidylglycerol and deprotonate probe F. Consequently, HEPES can significantly modify the acid−base properties of the negatively charged lipid membrane and thus affect the properties of phenolic compounds and other weak acids bound to lipid vesicles or cell membranes. It follows that HEPES buffer should be used with care in biomembrane studies.

Introduction

3-Hydroxyflavone (3HF) and 3-hydroxychromone derivatives present a considerable interest in the design of multiparametric fluorescence probes with a wide range of applications for studies of intermolecular interactions in liquids as well as in biological systems. Their attractiveness stems from an excited-state proton transfer (ESIPT) reaction they exhibit,[1] which results in two emission bands reflecting the presence of four states, the normal ground state (N), the normal excited state (N*), the tautomer excited state (T*), and the tautomer ground state (T) (which is a metastable ground state accessible only from the T* state). A solvent-dependent dual emission can be observed for other dyes exhibiting ESIPT reaction, but according to literature data, 3HF is the unique case in which its appearance is not connected with conformational isomerizations. The emission spectra from N* and T* forms are highly intensive bands well separated on the wavelength scale. Moreover, the presence at the 4′ position of 3HF of an electron donor dialkylamino group makes these dyes strongly solvatochromic.[2] It results that the interactions with their environment produce strong perturbations in the equilibrium between the excited-state tautomeric forms, leading to dramatic changes in the relative intensities of the two emission bands.[3] In addition, in basic conditions the 3-OH group can dissociate to yield a ground-state (A) and an excited-state (A*) anionic form.[4]

Due to their extreme sensitivity to solvent polarity and hydrogen bonding with protic solvents,[5] these probes are used as sensors for polar/protic impurities in nonpolar solvents[6] and for the presence of water molecules in reverse micelles.[7] These dyes are also strongly electrochromic, that is, sensitive to the strength and direction of local electric fields.[8] This allows these dyes to be used for the determination of dipole potential in biological membranes.[9] Moreover, we recently demonstrated that it is possible to simultaneously determine both polarity and hydration in phospholipid bilayers by using 4′-(dimethylamino)-3-hydroxyflavone (probe F).[10] This possibility is due to the presence of two forms of probe F at different locations in the bilayer: an H-bond-free form with a relatively deep location and displaying the classical two-band emission due to ESIPT reaction, and an H-bonded form with a shallower location and displaying a single-band emission with no ESIPT. The *local* polarity was estimated from the two-band spectra of the H-bond-free form, while the local hydration was estimated from the relative contribution of the two forms. This behavior of probe F in lipid vesicles was confirmed for other 3HF derivatives.[11] Such an analysis implies the determination with a good accuracy, through the deconvolution of the emission spectra, of a number of parameters, namely, the emission wavelength maxima (λ_{N*},

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λ_{T^*}, and λ_{H-N^*}) and the intensities (I_{N^*}, I_{T^*}, and I_{H-N^*}) of H-bonded and H-bond-free forms.

However, we were confronted in some cases, mainly in the study of vesicles made from phosphatidylglycerol lipid species, with an unexpected behavior of probe F and some other 3HF derivatives: a new band appeared at longer wavelengths of both the excitation and emission spectra. Selective excitation of this new absorption band strongly modifies the emission spectra, with the appearance of a new band between the N* and T* ones. The present work was aimed to analyze the origin of these additional absorption and emission bands. A screening of experimental conditions allowed us to attribute this effect to a specific interaction of probe F with the buffering 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) molecules in the presence of phosphatidylglycerol polar heads.

Materials and Methods

Egg yolk phosphatidylglycerol (EYPG) and HEPES (>99.5%) were purchased from Sigma. Probe F was synthesized and purified as previously described.[12] Large unilamellar vesicles (LUVs) were obtained by the extrusion method as previously described.[10,11] LUVs were labeled by adding an aliquot (generally 2 μL) of probe stock solution (2 mM) in dimethyl sulfoxide to 2 mL solutions of vesicles. The fluorescence spectrum was recorded a few minutes after addition of the probe, since the kinetics of binding are rapid. Either 15 mM HEPES or 15 mM PCB (phosphate−citrate−borate, 5 mM concentration for each anion) buffer was used in the experiments.

pH measurements were carried out on a 713 pH meter (Metrohm). Absorption and fluorescence spectra were recorded on a Cary 4 spectrophotometer (Varian) and a FluoroMax 3.0 (Jobin Yvon, Horiba) spectrofluorometer, respectively. Time-resolved fluorescence measurements were performed with the time-correlated, single-photon-counting technique using the frequency-doubled output of a Ti−sapphire laser (Tsunami, Spectra Physics), pumped by a Millenia X laser (Tsunami, Spectra Physics).[3] The excitation wavelength was set either at 427 nm or at 474 nm. The fluorescence decays were collected at the magic angle (54.7°) of the emission polarizer. The single-photon events were detected with a microchannel plate Hamamatsu R3809U photomultiplier coupled to a Philips 6954 pulse preamplifier and recorded on a multichannel analyzer (Ortec 7100) calibrated at 25.5 ps/channel. The instrumental response function was recorded with a polished aluminum reflector, and its full width at half-maximum was 50 ps. In the present case, the time-resolved fluorescence decay curves can be classically described by a multiexponential decay:[13]

$$I_i(\lambda) = \frac{\alpha_i(\lambda)\tau_i I(\lambda)}{\sum_j \alpha_j(\lambda)\tau_j} \qquad (1)$$

where α_i are preexponential coefficients and τ_i lifetime values. The data were analyzed by the maximum entropy method (MEM) using the Pulse 5.0 software.[14] The goodness of the fit was evaluated from the χ^2 values, the plots of the residuals, and the autocorrelation function.

Results

Absorption and Fluorescence Steady-State Experiments. Absorption and excitation spectra of probe F in organic solvents[5] and lipid vesicles[10,15] commonly appear as one band with a maximum in a rather narrow range, namely, between 390 and 415 nm. Absorption is driven by parameters such as electronic polarizability (refractive index) and hydrogen-bonding donor ability.[5] However, we found that the absorption spectra of probe F, embedded in EYPG vesicles in 15 mM HEPES buffer at pH between 6 and 11, present, besides the main band with its maximum around 410 nm as in lipid vesicles,[10,15] an additional band appearing as a shoulder at ca. 450 nm (Figure 1A). The relative intensity of this new additional band increases with pH, showing an isosbestic point in the absorption spectra around 427 nm. This shows that the new band corresponds to a new and single ground-state species that may be assigned to the anionic form of the dye, commonly detectable in the long-wavelength region of the spectrum.[4,16]

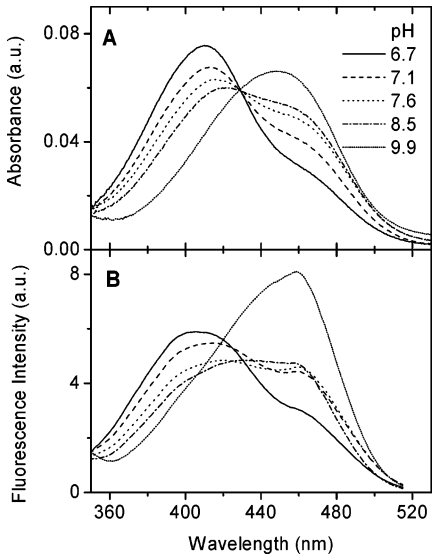

Figure 1. Absorption (A) and excitation (B) spectra of probe F in EYPG vesicles at different pHs in 15 mM HEPES buffer. Excitation spectra were recorded at 530 nm as emission wavelength.

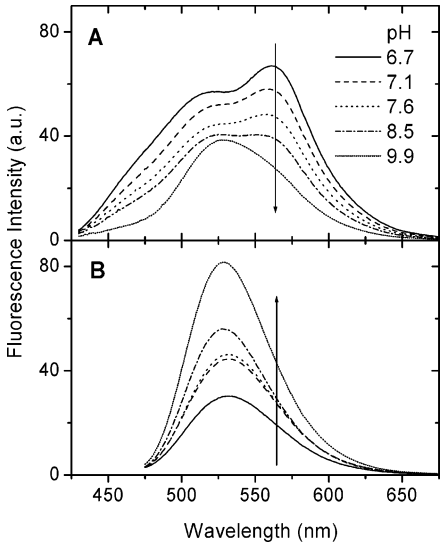

Figure 2. Emission spectra of probe F in EYPG vesicles in the pH range between 6 and 11 in 15 mM HEPES buffer: (A) excitation wavelength 400 nm, (B) excitation wavelength 460 nm.

Excitation of the sample at 400 nm results, as usually, in a dual-emission spectrum corresponding to ESIPT reaction (Figure 2A). In contrast, drastic changes in the emission spectrum are observed with an excitation at 460 nm, since only a single-band emission spectrum with a maximum around 530 nm appears (Figure 2B). The spectrum centered at 530 nm may be attributed to the photoselection of the anionic species by the

460 nm excitation wavelength. This conclusion is supported by the pH dependence of the fluorescence spectra recorded with excitation at 400 nm. Indeed, the fluorescence intensity of the long-wavelength band decreases strongly in this pH range, leading, at pH greater than 8, where the ground state is expected to be fully deprotonated, to a single-band spectrum with a maximum at approximately 535 nm. Thus, the fluorescence spectrum of probe F in EYPG vesicles in HEPES buffer presents a rather complex nature. Besides the emission of H-bond-free (nonhydrated) N* and T* forms and the emission of a H-bonded (hydrated) H−N* form,[10] the emission of an anionic form can also be observed. It should be noticed that the position of the emission maximum of the H−N* form, which was detected in different lipid vesicles (ca. 527 nm),[10] is very close to that observed for the anionic form (530 nm) in the present study. Therefore, it is difficult to estimate directly the contribution of each of these forms to the observed fluorescence spectra in the case of EYPG vesicles. Meanwhile, since in vesicles composed of other phospholipids[10] we did not detect any anionic form in the excitation spectra, we can conclude that the contribution of the ground-state anionic form is important only in the case of EYPG vesicles.

For excitation spectra recorded at 530 nm, the maximum emission wavelength of the anionic species (Figure 1B) presents an evolution with pH similar to that of the absorption spectra (Figure 1A), confirming that the anionic species is fluorescent, with an excitation maximum close to 460 nm. It should be noticed that, at high pH (9.9), the excitation maximum is ca. 10 nm red-shifted with respect to the corresponding absorption maximum. This suggests that the quantum yield of the anionic form is probably higher than that of other forms of the dye. Furthermore, at high pH we cannot neglect the presence of the anionic form of the dye F free in buffer, which, according to our data, exhibits an absorption maximum at 427 nm. Evidently, it is less emissive than the anionic form bound to the lipids (see time-resolved data below) and therefore contributes mostly to the absorption spectrum, shifting the observed absorption maximum to the blue with respect to the corresponding excitation maximum (Figure 1).

Some features deserve special attention. First, the absorption by the anion is observed even at neutral pH (Figure 1A). Second, according to the present data, the anionic form of probe F in water exhibits an absorption maximum at 427 nm, which is significantly blue-shifted as compared to that in EYPG vesicles in the presence of HEPES buffer (ca. 460 nm). Owing to the present data, we made the assumption that these unexpected features of the anionic form of dye F in EYPG vesicles (appearance at neutral pH, red shift of the absorption maximum) result from the presence of HEPES molecules.

To check this hypothesis, we performed the same series of experiments (excitation and emission spectra vs pH) for EYPG vesicles prepared in PCB buffer instead of HEPES. Interestingly, in PCB buffer, no additional band is detected in the excitation spectra recorded at 530 nm within a pH range between 6 and 9 (Figure 3). The position of the excitation maximum is significantly modified only at pH around 10 and above, shifting from 407 to 425 nm. Moreover, we observe the long-wavelength excitation band as a limited shoulder at 460 nm only at high pHs, whereas in HEPES it appears as a large band already at neutral pHs (Figure 1A). In complete agreement with these excitation spectra, the corresponding emission spectra recorded with excitation at 400 nm present a typical two-band profile (Figure 4A), with a complex broad short-wavelength band in line with those obtained with other kinds of vesicles.[10] The

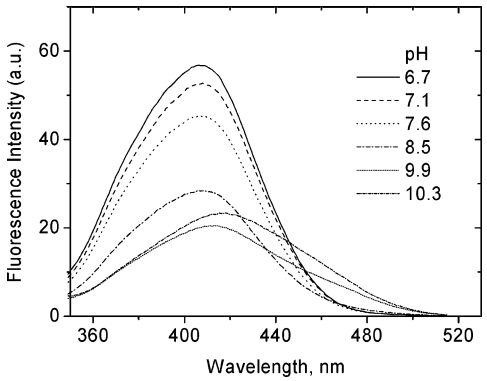

Figure 3. Excitation spectra of probe F in EYPG vesicles vs pH, in PCB buffer. The emission wavelength was 530 nm.

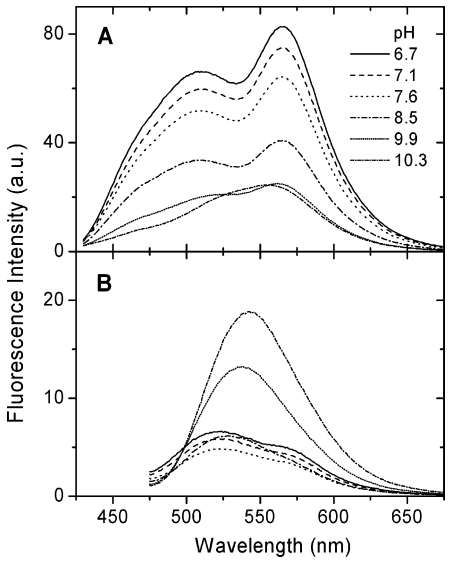

Figure 4. Emission spectra of probe F in EYPG vesicles in the pH range between 6 and 11 in 15 mM PCB buffer: (A) excitation wavelength 400 nm, (B) excitation wavelength 460 nm.

increase in pH results only in a decrease in intensity with almost no changes in the emission profile. A significant modification of the emission spectrum is observed only at pH greater than 10, where the anion formation results in a single-band emission spectrum with its maximum around 540 nm. Thus, in PCB buffer, both excitation and emission spectra obtained at pH greater than 10 can be easily assigned to the anionic species of the dye normally occurring at these high pH values.

Further information is obtained from the comparison of the fluorescence spectra in both buffers obtained with excitation at 460 nm. In HEPES buffer, only a single-band spectrum stable in the whole range of pH with a maximum at 540 nm is observed (Figure 2B). In contrast, in PCB buffer, a low-intensity dual emission is observed up to pH 8 and the single-band emission appears only at higher pH, with its maximum shifting from 530 to 545 nm (Figure 4B). These observations prove that, in the case of PCB buffer, even in the condition of photoselection of the anionic form (excitation at 460 nm), the excitation of this anionic form is not exclusive so that the normal form is observed in emission up to pH 8 and probably remains present enough at higher pH to induce the relative blue shift of the maximum of the anionic form spectrum. Thus, in PCB buffer in opposition to HEPES buffer, the anion formation of dye F occurs normally at the high pH range typical for phenols. Furthermore, the emission maximum of the anionic band in PCB buffer (Figure 4B) corresponds well to that observed in water (446 nm).

TABLE 1: Time-Resolved Data of the Anionic Form of Probe F in Buffers at pH 10.3[a]

	λ_{exc}, nm	τ, ns		λ_{exc}, nm	τ, ns
HEPES	427	0.93	PCB	427	0.91
	474	0.96		474	0.93

[a] λ_{exc} = excitation wavelength; τ = fluorescence lifetime.

These results prompted us to consider that, in HEPES buffer, the behavior of the probe is unusual since the new excitation band appears already at neutral pH with its maximum red-shifted by 20−30 nm from the expected values. Thus, it seems that HEPES molecules induce the appearance of the anionic form of probe F.

However, since it appeared quite surprising that the same anionic species could emit fluorescence at both neutral and highly basic pH values, more information was needed to confirm the conclusions drawn from the steady-state experiments. For this reason, we performed time-resolved fluorescence experiments in the same conditions.

Time-Resolved Fluorescence Experiments. Time-decay measurements were performed at two excitation wavelengths, 427 and 474 nm. The first excitation wavelength allows excitation essentially, but not only, of the normal N form of probe F, while the latter excites selectively the anionic form. First, time-resolved fluorescence experiments were performed with probe F dissolved in HEPES or PCB buffers. At neutral pH, probe F is nonfluorescent, while at high pH (10.3), its anionic form is highly fluorescent, exhibiting a single-band emission spectrum with an intensity maximum at 555 nm similar to that of the anionic form in the presence of EYPG vesicles. The fluorescence decay of the anionic form of probe F is monoexponential, confirming the absence of any excited-state reaction. Moreover, the single lifetime value was found to be independent of the excitation wavelength and the buffer (Table 1), suggesting no direct interaction of the dye with the buffer molecules. Second, the fluorescence decay curves of probe F embedded in EYPG large unilamellar vesicles were analyzed at two pH values (6.1 and 10.3), in HEPES and PCB buffers (Table 2). The excitation wavelength was 427 nm, and the decay curves were recorded at three different emission wavelengths: 480, 520, and 580 nm.

At pH 6.1, in both HEPES and PCB buffers, fluorescence decays recorded at the wavelengths corresponding to the emission of the neutral forms of the dye (480 nm for the normal N* form and 580 nm for the tautomer T* form) can be described by two components, suggesting the presence of two emissive species, in line with the bimodal distribution of probe F in lipid bilayers.[10] Indeed, it has been shown that probe F locates at two different depths in the bilayer, so that the hydrated species (H-bonded with water exhibiting H−N* emission[10]) of the dye locate at the interface while the water-free species are located deeper in the bilayer. According to our previous lifetime data in aprotic and protic solvents,[3,17] the short-lived lifetime component ($\tau_1 = 0.70-0.78$ ns) may be assigned to the emission of the deeply located water-free species (N* and T* emission), while the longer component ($\tau_3 = 2.1-2.5$ ns) may correspond to the hydrated species (H−N* emission). At pH 10.3 in HEPES buffer, a new component, τ_2 (0.91−0.96 ns), appeared at 580 nm. This new component may be attributed to the excited anionic form of probe F in the bulk water, since it corresponds to the lifetime value measured for probe F in buffers as shown in Table 1.

The nature of the buffer becomes important when the fluorescence decay is recorded at 520 nm, close to the emission maximum of the anionic form. In HEPES at both low and high pH, we observe a new component, τ_4, with a long-lived lifetime (3.6 ns), that is not detectable in PCB buffer (Table 2). This new component could be assigned to the emission of the anionic species that was inferred from the steady-state spectra at neutral pH in HEPES. To check this, we excited probe F at 474 nm at pH 10.3, to ensure the selective excitation of the anionic species. The fluorescence decay for both buffers is described by two lifetime components (Table 3). Evidently, the short component corresponds to the emission of the anionic free form of the dye in buffer (see Table 1), while the long component may be assigned to anionic species bound to the lipid bilayers. Importantly, the longest lifetime observed in this case (3.8 ns) matches closely the τ_4 component detected with excitation at 427 nm, further indicating that the latter corresponds to the emission of the anionic form of the dye in lipid bilayers.

Interestingly, in the case of the 474 nm excitation wavelength, probe F embedded in EYPG vesicles exhibits the same long-lived lifetime component ($\tau \approx 3.8$ ns) in both HEPES and PCB buffers, while at a 427 nm excitation wavelength this component ($\tau_4 = 3.6$ ns) is observed only in HEPES (Table 2). It is likely that this form is present only in a very small proportion in PCB buffer at pH 10.3, so that only a selective excitation at 474 nm allows resolving it. This may explain why, in this case, the anionic form of the dye appears only as a shoulder around 460 nm in the excitation spectra (Figure 3).

In addition, we detected very short lifetime components (<0.5 ns) which appeared when the samples were excited at 427 nm (not shown). Thus, at 480 nm emission, we observe one component (0.15−0.18 ns) with positive amplitude, while at 580 nm we detect two components (0.05−0.07 and

TABLE 2: Time-Resolved Data of Probe F in EYPG LUVs, Obtained with Excitation at 427 nm[a]

LUV/buffer	pH	$\lambda_{em.}$, nm	τ_1, ns	α_1	τ_2, ns	α_2	τ_3, ns	α_3	τ_4, ns	α_4	χ^2
LUV/HEPES	6.1	480	0.70	0.78			2.08	0.22			1.0
		520	0.77	0.48			2.13	0.40	3.64	0.12	1.0
		580	0.76	0.10			2.15	0.90			1.1
	10.3	480	0.72	0.66			2.25	0.34			1.2
		520			0.96	0.80	2.36	0.08	4.18	0.12	1.3
		580	0.74	0.29	0.93	0.39	2.49	0.32			1.0
LUV/PCB	6.1	480	0.71	0.75			2.09	0.25			1.0
		520	0.78	0.54			2.44	0.46			1.2
		580	0.74	0.38			2.32	0.62			1.1
	10.3	480	0.72	0.75			2.07	0.25			1.0
		520	0.77	0.31	0.91	0.20	2.54	0.49			1.1
		580	0.74	0.22	0.91	0.15	2.42	0.63			1.1

[a] λ_{em} = emission wavelength at which the decay was recorded; τ_1, τ_2, τ_3, and τ_4 = fluorescence lifetimes; α_1, α_2, α_3, and α_4 = preexponential coefficients; χ^2 = statistical criterion for the goodness of fit. The short lifetimes, <0.5 ns, are not presented (see the text).

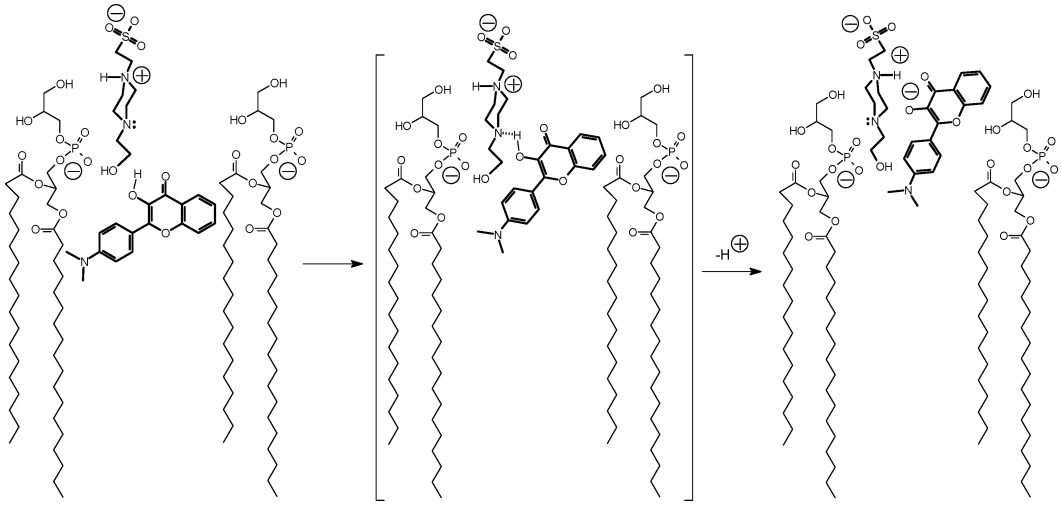

Figure 5. Suggested scheme of the anion formation of probe F in EYPG vesicles induced by HEPES molecules.

TABLE 3: Time-Resolved Data of Probe F Obtained at pH 10.3 in EYPG Vesicles, Obtained with Excitation at 474 nm

	τ_1, ns	α_1	τ_2, ns	α_2	χ^2
LUV/HEPES	1.00	0.59	3.87	0.41	1.1
LUV/PCB	0.99	0.60	3.77	0.40	1.1

[a] Symbols as in Table 2. The emission wavelength was 520 nm.

0.38−0.40 ns) with negative amplitude. The presence of components with a negative amplitude at the wavelength of the T* emission is a direct demonstration for the ESIPT process.[3,17,18] However, we do not have a clear explanation of why two negative components are observed. We could speculate that the short component (0.05−0.07 ns) corresponds to the fast ESIPT of the nonhydrated form of the dye, while the longer component (0.38−0.40 ns) corresponds to the slow ESIPT of its hydrated form. This issue will require additional systematic time-resolved studies in lipid vesicles of different compositions, which are currently in progress.

Thus, the present time-resolved data allow us to identify five emissive forms of dye F in EYPG vesicles at a high pH of the HEPES buffer. Namely, we observe three nonanionic forms, namely, N* and T* nonhydrated forms exhibiting the same lifetime ($\tau_1 = 0.70-0.78$ ns) and the hydrated H−N* form ($\tau_3 = 2.1-2.5$ ns), which are in line with our previous data in different lipid vesicles.[10] The other two forms are anionic forms emitting from bulk buffer ($\tau_2 = 0.91-0.96$ ns) and from vesicles ($\tau_4 = 3.6$ ns). The largest lifetime of the latter species supports our conclusion, based on comparison of absorption and excitation spectra, that the anionic form in vesicles exhibits the highest quantum yield compared with the other species. This form is present in both buffers at high pH, while at neutral pH it is generated only in the case of HEPES buffer.

Discussion

The present data demonstrate that, in anionic lipid EYPG vesicles in the presence of HEPES buffer, dye F exists in different forms and locations. The neutral forms of dye F (nonhydrated N* and T* and hydrated H−N*) were previously well characterized by steady-state fluorescence measurements in different lipid vesicles.[10] The present time-resolved data demonstrate that these forms do exist and exhibit lifetimes comparable to those in corresponding organic solvents. Thus, the H-bond-free forms of dye F in different aprotic organic solvents exhibit lifetimes in the range 0.3−1 ns.[3,17,18] These values are close to those observed for the nonhydrated forms of dye F in lipid vesicles ($\tau_1 = 0.70-0.78$ ns). According to our recent data,[17] the H-bonded form of the dye in protic solvents shows a lifetime in the range 0.6−2 ns. The observed value of the lifetime of the H-bonded form in lipid vesicles ($\tau_3 = 2.1-2.5$ ns) is somewhat higher, which can be connected with the relatively high microviscosity at the membrane interface.

The time-resolved fluorescence experiments also reveal new species with a lifetime value of ca. 4 ns observed for dye F in EYPG vesicles prepared in HEPES buffer in the pH range from 6 to 11. This species is present in PCB buffer only at high pH as a minor component. Evidently, this species corresponds to the anionic form of probe F bound to EYPG bilayers. Furthermore, for both buffers at high pH, we observe a contribution from the emission of the anionic form of the free dye in the buffer.

However, two questions remain pendant. Why does the anionic form of the dye already appear at neutral pH in EYPG vesicles in the presence of HEPES buffer, and why do these species absorb at much longer wavelengths as compared with the free anion in buffer? It has been reported that polar media such as water, alcohols, or formamide induce the formation of 3HF anion in its ground state even in the absence of any basic agent.[4,16] Thus, we suppose that, in the case of probe F bound at the polar charged interface of EYPG vesicles, anion may form even at neutral pH values. In this respect, HEPES molecules play a deprotonating role that cannot be played by the ion species composing the PCB buffer. Unlike the latter, HEPES at neutral pH values is a zwitterionic molecule with a positive charge on one of the protonated nitrogens. Due to this positive charge as well as to the presence of lipophilic hydrocarbon substituents, HEPES molecules may be adsorbed at the interface of an EYPG lipid bilayer in a rather ordered manner (Figure 5) and play the role of a base inducing the deprotonation of the dye. Thus, HEPES molecules in the presence of EYPG vesicles work as an amphiphilic deprotonation reagent operating at the level of the bilayer interface. As previously reported for another phenolic dye, 4-heptadecyl-7-hydroxycoumarin, an additional factor that may induce the ground-state deprotonation of probe F is the strong electric fields at the surface of a bilayer composed of anionic phospholipids.[19] This could explain why, for vesicles composed of neutral lipids, we could not detect any anionic form at neutral pH in HEPES buffer. Finally, these surface charge effects could also explain the anomalous red-shifted

absorption of the anionic species of dye F at the membrane interface, in the presence of both buffers.

Conclusion

The appearance of a new band, strongly shifted to the red, in both absorption and excitation spectra of probe F embedded in EYPG vesicles was an unexpected phenomenon. It seems to be caused by the presence of HEPES buffer molecules and does not appear with the phosphate−citrate−borate buffer. According to time-resolved fluorescence data, this new band has been attributed to the anionic form of probe F bound at the interface of the lipid bilayer. This anion forms even at neutral pH due to the deprotonating properties of HEPES molecules able to bind at the bilayer interface to the negatively charged polar heads of phosphatidylglycerol. Our results show that HEPES can induce an important modification of the acid−base properties of the negatively charged lipid membrane. Therefore, HEPES can modify the behavior of phenolic compounds and other weak acids bound to lipid vesicles or cell membranes. This should lead to some precautions when using HEPES as a buffer in biomembrane studies.

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