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Neutral fluorescence probe with strong ratiometric response to surface charge of phospholipid membranes

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Abstract We report on dramatic differences in fluorescence spectra of 4′-dimethylamino-3-hydroxyflavone (probe F) studied in phospholipid membranes of different charge (phosphatidyl glycerol, phosphatidylcholine (PC), their mixture and the mixture of PC with a cationic lipid). The effect consists in variations of relative intensities at two well-separated band maxima at 520 and 570 nm belonging to normal (N*) and tautomer (T*) excited states of flavone chromophore. Based on these studies we propose a new approach to measure electrostatic potential at the surface layer of phospholipid membranes, which is based on potential-dependent changes of bilayer hydration and involves very sensitive and convenient ratiometric measurements in fluorescence emission. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Membrane surface charge; Fluorescent ratiometric probe; Phospholipid vesicle; 3-Hydroxyflavone; Excited state proton transfer

1. Introduction

In biological membranes and their phospholipid models a steep gradient of electrostatic potential exists at the lipid–solvent interface at distances of several Å [1–4]. This electrostatic field gradient depends dramatically on the properties of the surface: the presence of lipid heads, their charges and the ionic atmosphere around the membrane. Indeed, the interface is not merely a thin layer separating the hydrophobic and the aqueous phases, but an interface region having a non-uniform and complex structure and properties. For example, the headgroup area formed by zwitterionic phosphatidylcholine (PC) molecules can be divided into two regions, a deeper one corresponding to the negatively charged phosphate groups (1.8 nm thick) and a more superficial one corresponding to the positively charged cholines (2 nm thick) [4]. These features are demonstrated clearly in computer modeling of membrane dynamics [5,6]. The relative importance of these two regions could be evaluated by replacing PC by some anionic lipid like phosphatidyl glycerol (PG), or by inserting cationic lipids or surfactants into the bilayer. Meantime, the experimental possibilities for probing these charge distributions are very limited. The charged fluorescence probes (e.g. ANS [7,8]) can not protrude deeply into the bilayer, while the neutral ones (e.g. Laurdan or Prodan [9,10]) are not sensitive enough to the effects of charge. A substantial step forward was made with the introduction of electrochromic probes [11–13], but their response is a gradual shift of excitation and emission spectra with ratiometric response (differential response at two characteristic wavelengths) not exceeding 10%.

Ratiometric response in emission spectra is a very desirable property for many applications. This is the reason why 3-hydroxyflavone derivatives possessing this property attract increasing attention as prospective candidates for fluorescence sensors and probes. Their important feature is the presence of two bands in fluorescence emission spectra that belong to normal (N*) and tautomer (T*) excited states of flavone chromophore [14]. The T* form appears as a result of excited state intramolecular proton transfer reaction. This reaction is very sensitive to different perturbations produced by the chromophore surrounding on its π-electronic system and can involve solvent–chromophore dipole–dipole interaction and hydrogen bonding [15,16]. Thus, an amplification effect can be generated, which results in the coupling of relatively small spectral shifts of initially excited N* form with more significant (and often dramatic) redistribution of relative intensities between N* and T* fluorescence emission bands. These unique properties have been already used in the studies of detergent micelles [17], reverse micelles [18] and phospholipid membranes [19,20]. Thus, the effects of the presence of cholesterol and of the addition of ethanol have been described [19] with the observation of significant changes in N*/T* ratios.

In the present report we demonstrate a dramatic variation of probe F fluorescence spectra recorded in vesicles consisting of phospholipids of different charge. This is a rather unexpected finding in view of the low-polar character and neutral charge of this probe. The study of effects of added cationic detergents, increase of ionic strength, as well as experiments in model binary solvent mixtures allow to suggest a new mechanism of this response.

2. Materials and methods

All the chemicals were of Aldrich or Sigma quality, and of spectro-

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Abbreviations: CTAB, cetyl trimethylammonium bromide; PG, phosphatidyl glycerol; PC, phosphatidylcholine; EYPC, egg yolk PC; EYPG, egg yolk PG; DMTAP, *N*-[1-(2,3-dimyristoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium tosylate salt

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scopic grade for the solvents. Flavone F was synthesized and purified as described elsewhere [21]. Titration of ethyl acetate with water was performed by addition of 4 μl of water aliquots to 2 ml of 5×10^{-6} M solution of F in ethyl acetate, in order to increase gradually concentration of water by 0.11 M increments up to a final concentration of 1.1 M (2%, v/v). Vortex of the solution was needed to reach homogeneity.

Large unilamellar vesicles (0.11–0.12 μm in diameter) were obtained by extrusion on polycarbonate filters as previously described [22]. They were made either of egg yolk PC (EYPC) and/or PG (EYPG), both from Sigma and of the cationic lipid *N*-[1-(2,3-dimyristoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium tosylate salt (DMTAP), a gift from Dr. Heissler (Strassbourg). Absorption spectra were performed on Cary 3 Bio (Varian) spectrophotometer. Fluorescence spectra in solvents were recorded on Quanta Master (PTI) and those in vesicles on SLM 48000 (SLM-Aminco) spectrofluorometers.

Deconvolution of fluorescence spectra into two Lorentzian components was performed using Microcal Origin 5.0. No dependence of deconvolution results was found on setting initial parameters (band maxima and widths).

3. Results

Fig. 1 demonstrates the major result of this work. When the probe F is incorporated into large unilamellar vesicles of different composition, its fluorescence spectra exhibit dramatic differences. Decrease of negative charge and increase of positive charge per lipid molecule from −1 (for PG) to +0.5 (for DMTAP/PC mixture) decreases sequentially up to 2.2 times the contribution of N* band relatively to T* band. Such a remarkable spectroscopic effect dependent on phospholipid composition and charge to our best knowledge has not been reported with any fluorescence probe so far. Remarkable also is the dynamic range of this effect, which extends with the same regularity in the direction of both negative and positive charges.

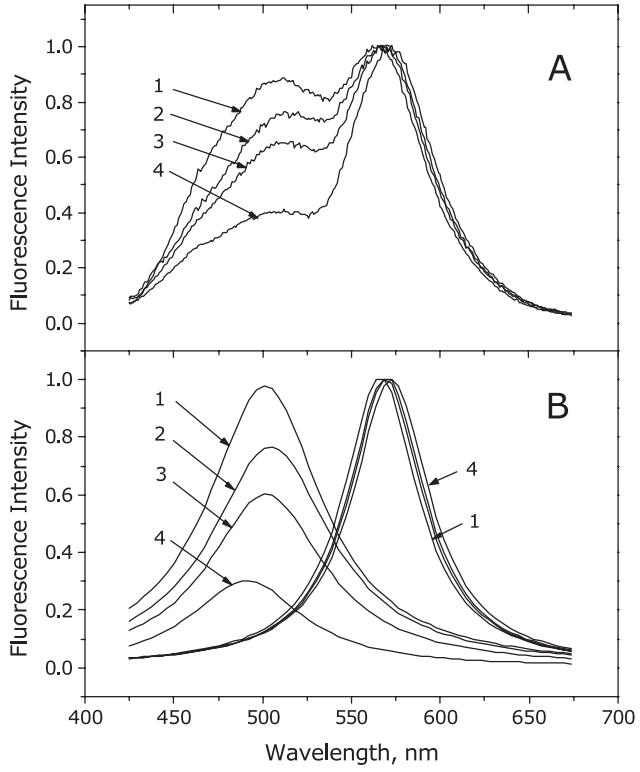

Fig. 1. Fluorescence spectra of flavone F in large unilamellar vesicles, which differ in surface charge (A) and their deconvolution into two components (B). Vesicles are composed of: EYPG (1), EYPG/EYPC, 1:1, mol/mol (2), EYPC (3) and DMTAP/EYPC, 1:1, mol/mol (4). The spectra were normalized at long-wavelength maximum. Excitation wavelength: 400 nm. Lipid to probe ratio: 100. Buffer: HEPES 15 mM, pH 7.4.

The correspondent excitation spectra recorded for different lipids and lipid mixtures at N* and T* fluorescence band maxima (not shown) superimpose totally (with the exception in the case of PG of almost negligible additional contribution to intensity at long-wavelength edge). Therefore, for all studied cases both N* and T* emission bands originate from the same ground state species, as it should be for the excited state reaction. Moreover, the polarity of the probe environment in the binding sites in vesicles of different composition is approximately similar. This conclusion is also supported by the absence of observable shifts in position of N* band in fluorescence spectra, which is known to be very sensitive to solvent polarity [15,16,21]. The results of deconvolution of spectra into two components (Fig. 1B) show that the positions of N* form do not change from PG (501 nm) to PC (502 nm) vesicles, and the shift by 10 nm to shorter wavelengths is observed only in the case of DMTAP/PC mixture. The somewhat less polar probe environment in the latter case may provide an additional contribution to ratiometric effect.

The electrostatic potential and charge at the membrane–solvent interface can be modulated by different means, and it is interesting to see what could be the effects on fluorescence spectra of probe F. Cationic detergent cetyl trimethylammonium bromide (CTAB) can be incorporated into membrane with fixation of its positively charged polar head [23]. According to our data, the effect of CTAB is apparent in the case of vesicles made of PG (Fig. 2). In this case the relative intensity of N* band goes down and the fluorescence spectra approach in shape the spectrum observed in PC vesicles throughout the whole recorded wavelength range. The effect of CTAB addition is non-linear and tends to saturation at the molar ratio of CTAB to PG of 40%. Addition of CTAB to the PC/PG mixture (1:1, molar) results in a similar effect, but of smaller magnitude. It is not observed for PC vesicles, where the addition of CTAB does not produce any spectroscopic changes. The partition of CTAB molecules into anionic vesicles made of PG is important, so that they neutralize by electrostatic interactions the negatively charged phosphate groups. The partition into vesicles made of zwitterionic PC is much lower [23] and in this latter case the electrostatic environment of the probe may not be significantly disturbed. The increase of KCl concentration to 140–175 mM produces the changes of probe F fluorescence spectra in PG vesicles in the same direction, but their magnitude is smaller (Fig. 2). The origin of these changes is probably the formation of diffuse layer of K^+ ions in the aqueous phase that partially compensates the surface charge. In PC vesicles the effects of KCl were not observed.

In order to find rational explanation of observed data, we studied some solvent-dependent properties of probe F in model neat solvents and solvent mixtures. Fluorescence spectra of flavone F and its analogs are known to be extremely sensitive to properties of solvents [15,16,21]. The increase of polarity and hydrogen bonding ability of the solvent leads to dramatic increase of N* band relatively to T* form. This is due to dielectric stabilization of N* form, which possesses higher dipole moment. We observe (Fig. 3A) that the range of ratiometric response for probe F is rather narrow. It covers only

aprotic solvents with polarities roughly between ethyl acetate (with solvent polarity index $E_T(30) = 38.1$ [24]) and acetonitrile ($E_T(30) = 45.6$). For toluene with lower polarity, N* band is of negligible intensity, while in solvents with polarity higher than acetonitrile and in all tested protic solvents (e.g. alcohols) the N* band is observed only. For the studied phospholipid membranes (see Fig. 1) it is not only the ratio of N* and T* forms but also the spectral position of the N* form that fits within the solvent range ethyl acetate–chloroform–acetonitrile. This fact suggests that the probe surrounding in the studied phospholipid vesicles is aprotic and relatively low-polar. This conclusion is in line with recent observations on the behavior of probe F in dimyristoyl PC and dipalmitoyl PC liposomes [20].

If the probe is neutral and its environment in the membrane is low-polar, then what produces the variation of intensities between N* and T* forms? We decided to consider the possibility of complex formation between the probe and individual water molecules in hydrophobic membrane interior and to model this situation in binary solvent system. Fluorescence spectra of probe F were measured in ethyl acetate on the addition of small amounts of water (Fig. 3B). We observe a very strong gradual increase of N* form fluorescence intensity with the increase of water concentration. The effect is dramatic. Initially the probe in ethyl acetate contains in emission only a small amount of N* form. But when water concentration is increased up to 1.1 M (2%, by volume), the N* and T* forms become nearly the same in intensity. The ratio of N* and T* band intensities increases by 4.3 times. Simultaneously, a gradual shift of the N* band maximum to longer wavelengths is observed.

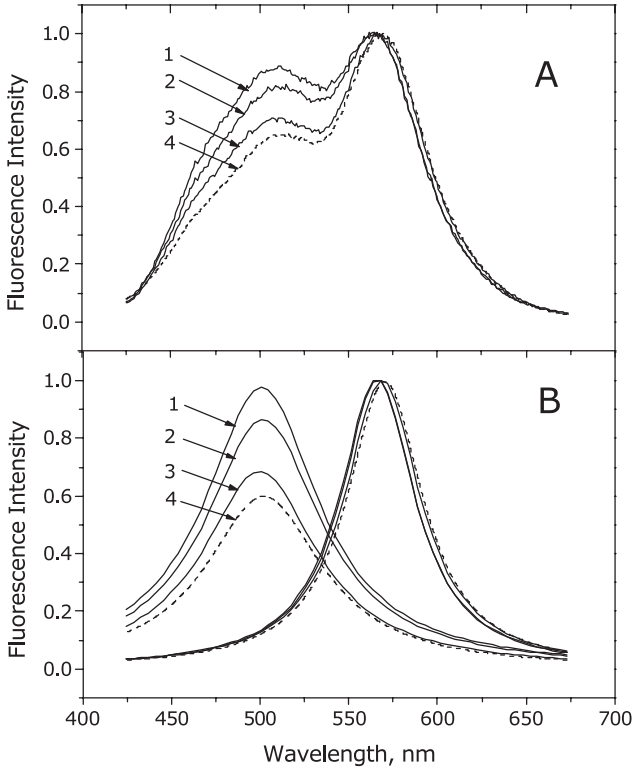

Fig. 2. Effect of CTAB on fluorescence spectra of flavone F in large unilamellar vesicles (A) and on two components produced by their deconvolution (B). Vesicles are composed of EYPG (1), EYPG in 175 mM KCl (2), EYPG+CTAB (1:0.2, mol/mol) (3) and EYPC (4). The spectra were normalized at long-wavelength maximum. Experimental conditions as in Fig. 1.

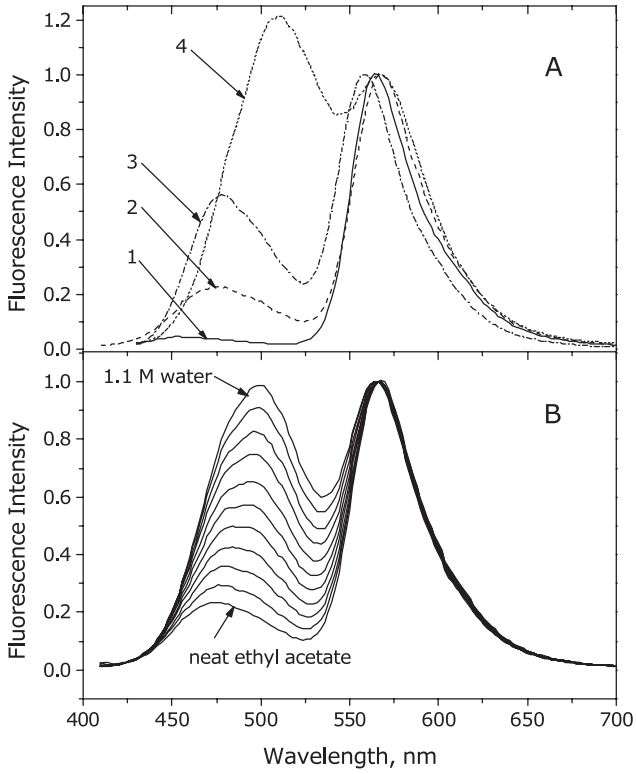

Fig. 3. Solvent effects on fluorescence spectra of flavone F. A: Flavone F in toluene (1), ethyl acetate (2), chloroform (3), and acetonitrile (4). Probe concentration 5×10^{-6} M. B: Effect of water in the range 0–1.1 M on fluorescence spectra of 5×10^{-6} M flavone F in ethyl acetate. Each addition of water was 0.11 M (0.2%, v/v). The spectra were normalized at long-wavelength maximum. Excitation wavelength: 400 nm.

The latter results demonstrate a possibility of strong connection between fluorescence of probe F and its hydration in low-polar environments. However, we could not expect complete similarity between the results obtained in phospholipid vesicles and in model solvents. Thus, the shifts in the position of N* band with the increase of water content (which is not observed in PC–PG vesicles) may be the result of more isotropic distribution and higher mobility of water in mixed solvent system.

4. Discussion

Design of ratiometric fluorescence probes for different biomembrane applications is a great challenge for the researchers. These probes are more convenient and feature greater precision than the probes operating by the change of emission intensity or lifetime. Their response is concentration-independent, and they produce visually observed change of emission color, which is important for microscopic applications. In order to become ratiometric the probe should exhibit some reaction in the excited state, which changes the energy of electronic transition and produces the wavelength-shifted fluorescence spectra.

The probe F was selected because our preliminary studies together with the literature data [20] showed that in the most abundant phospholipid, PC, it exhibits two well-separated N*

and T* maxima with almost equal intensities. This property is optimal for ratiometric response. It is of importance for the synthesis of new compounds with improved spectroscopic properties [25], which offer new possibilities in biomembrane research.

In the present work a series of lipids and their mixtures of different charge was studied, and the response of probe F to our surprise was found to be dramatically sensitive to the phospholipid charge and accordingly to the interface potential in bilayers formed from these phospholipids. The probe F is uncharged and low-polar. Therefore it should be bound rather deeply behind the charged biomembrane surface and should not have significant preference in binding to phospholipids of different types. Because of the absence of charge on F molecule its direct electrostatic interaction with phospholipid charges is not significant and should be limited to only a small electrofluorochromic effect [26]. Therefore, the observed dramatic variations of band intensities must be indirect in nature.

We can consider two models of these indirect effects: the probe relocation model and the differential hydration model. According to relocation model the probe molecules in bilayers formed of different lipids are positioned at different depths with their environment differing in polarity, molecular order or electric field. There is a steep gradient of polarity across the membrane surface (with the change of dielectric constant from 2 to 80) [27]. The non-charged probe F with its polar carbonyl and hydroxyl groups and a non-polar rest of the molecule can move towards the equilibrium occupying the position, which corresponds to free energy minimum with regards to this gradient. Meantime, the electrostatic factors are probably not sufficient for providing relocation of the probe. The change of the probe location and orientation in the ground state is low-probable in view of its small ground state dipole moment [28], while reorientations in the excited state can be ruled-out because of the observation of a strong static Red-Edge effect [20] demonstrating the absence of dipole-relaxational dynamics.

According to the differential hydration model, the binding sites in bilayers that possess different charge differ by access to hydration water. We expect the highest hydration for the case of PG vesicles due to repulsion of non-compensated negative charges of phosphate groups, which makes the membranes more available for water. In vesicles composed of zwitterionic PC these charges are partially compensated with choline groups, which condense the membrane and allow less access to water. Intermediate properties are expected for PC–PG mixture, since the two lipids are dispersed homogeneously. Finally, the addition of DMTAP lipid bearing positive ammonium group located at the same position as the phosphate groups of PC can produce the most efficient compensation of their negative charges with the resulting decrease of access to water of membrane interior.

Greater hydration of probe F should exhibit higher relative emission of N* form. The above presented results of our studies with probe F in ethyl acetate with the addition of small amounts of water illustrate this situation. In a related study [29] the addition of water to highly polar aprotic solvent acetone also resulted in dramatic changes of fluorescence spectra. Regarding the biomembrane research, the differential hydration model can also be supported by the available literature data. The water molecules are known to penetrate deeply into the bilayer and provide the hydration of phosphate and carbonyl groups [6,30]. The estimated hydration level is 4.0 water molecules per phosphate group and 1.0 per carbonyl group [4]. The studies of C = O stretching bands of two phospholipid ester carbonyl groups by IR and Raman spectroscopy [31,32] demonstrated higher hydration of these groups in PG than in PC, which is in line with the conclusion that can be made from our data based on differential hydration model. The NBD-based neutral probes respond to electrostatic membrane potential by variation of emission intensity [33] probably also by differential hydration effects. These probes are nearly non-fluorescent in water [33,34], and their differential quenching in membranes as a function of dipole potential may be due to variations in interaction with water molecules.

In conclusion, it appears from this study that 3-hydroxyflavone probe F can be a very sensitive tool to analyze the effects of biomembrane charge and achieve better understanding of an important and frequently debated [34–37] question on connection between electrostatic and hydration properties of phospholipid bilayer.

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