An Intramolecular Excimer Forming Probe Used To Study the Interaction of α-Lactalbumin with Model Membranes†

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ABSTRACT: The nonconjugated bichromophoric molecule 1,3-di(1-pyrenyl)propane shows, besides the pyrene monomer fluorescence, a structureless emission due to an intramolecular *exci*ted di*mer* (excimer). In the case of intramolecular excimer forming systems, the ratio of the emission intensities of excimer vs. monomer (I_E/I_M) is sensitive to changes in membrane structure as will be illustrated here. The present molecule is a useful probe to report on lipid–protein interactions, at least in our model system. We have introduced it into the hydrocarbon layer of dimyristoylphosphatidylcholine vesicles in order to study their interaction with α-lactalbumin (α-LA) as a function of pH and temperature. On the basis of steady-state fluorescence, kinetic, and energy transfer studies, we have found that, at pH 4, α-LA strongly interacts with the lipid bilayer. In steady-state fluorescence experiments, changes of the ratio I_E/I_M and shifts of the transition temperature have been observed, reflecting changes of membrane structure caused by interaction with α-LA. Kinetic studies of the rate of interaction of α-lactalbumin and measurements of energy transfer from excited tryptophan(s) to the fluorescent probe confirm the steady-state experiments. Our results agree with previously reported microcalorimetric, gel chromatographic, and fluorescence polarization studies.

Intermolecular excimer forming systems (e.g., pyrene) have been extensively used as probes of molecular mobility changes occurring in the hydrocarbon interior of phospholipid dispersions. These can be evaluated from the change of the ratio of emission intensities of excimer to monomer (I_E/I_M)[1] (Soutar et al., 1974; Galla & Sackmann, 1974). In contrast to intermolecular systems, the formation of intramolecular excimer is concentration independent, allowing the use of very low concentrations (1 μM) and thus minimizing the risk of perturbation of the phospholipid phase. Recently we have introduced an intramolecular excimer forming, nonconjugated, bichromophoric molecule: 1,3-di(β-naphthyl)propane as a new probe for measuring thermal phase transitions in aqueous dispersions of a variety of synthetic phospholipids (Dangreau et al., 1979).

A number of authors, especially Wang & Morawetz (1976), Zachariasse & Kühnle (1976), and Goldenberg et al. (1978), have reported that intramolecular excimer formation is sensitive to the bulk viscosity of organic solvents. This principle has been used to study the microviscosity (changes) of structured molecular systems such as micelles (Zachariasse, 1978; Emert et al., 1979; Turro et al., 1979). Zachariasse (1978) has introduced the term "microfluidity" instead of microviscosity to emphasize the difference between macroscopic viscosity and molecular mobility in microenvironments.

An important prerequisite that must be fulfilled by a fluorescent probe, which is to be used as a reporter on lipid–protein interactions, is that its excitation wavelength does not overlap with the absorption spectrum of proteins. A suitable probe, bis(1-pyrenylmethyl) ether, has been introduced by Georgescauld et al. (1980) to study the phase transitions in synthetic phospholipid membranes. However, to our knowledge, this probe has not been applied in lipid–protein interaction measurements.

We have synthesized 1,3-di(1-pyrenyl)propane (DPP), which has an excitation peak at 348 nm (Zachariasse & Kühnle, 1976). In the present work we have used this intramolecular excimer forming probe to study lipid–protein interactions.

As a model, the interaction of α-lactalbumin (α-LA) with dimyristoylphosphatidylcholine (DMPC) dispersions has been investigated. Our model protein has a variable behavior, depending on pH. As shown by microcalorimetric and gel chromatographic studies (Hanssens et al., 1980), α-LA behaves as a peripheral protein above its isoelectric point (pH 5.0), mainly adsorbing to the outer surface of the membrane. Below pH 5.0 however, α-LA extensively disturbs the lipid bilayer.

In this work we have first assessed the applicability of DPP to report on phase transitions in DMPC dispersions. Second, we have used DPP steady-state fluorescence to study the interaction of α-LA with DMPC vesicles at different pH values. In addition, we have followed the kinetics of interaction of α-LA with DMPC vesicles at T_t for different pH values. Finally, additional evidence for the strong interaction of α-LA with DMPC vesicles at acidic pH is provided by measuring the energy transfer from excited tryptophan(s) to DPP embedded in the hydrocarbon layer of the phospholipid phase.

The main conclusion is that—in our system—DPP is a useful probe to study lipid–protein interactions. It may have universal applications in other model proteovesicles and even in natural membranes (Melnick et al., 1981).

Experimental Procedures

Materials. α-LA from bovine milk is purchased from Sigma. It is used without further purification as in previously published studies (Hanssens et al., 1980; Herreman et al., 1981a,b). However, the purity of this product has been checked by gel filtration, $NaDodSO_4$–polyacrylamide gel electrophoresis, and isoelectric focusing. The batch used in our experiments contains at least 90% α-LA. We have identified two impurities as being β-lactoglobulin and serum albumin. The product does not contain measurable amounts of fatty acids, whether esterified or not. α-LA concentration

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[1] Abbreviations: I_E/I_M, ratio of intensities of excimer to monomer emission; α-LA, α-lactalbumin; DMPC, dimyristoylphosphatidylcholine; DPP, 1,3-di(1-pyrenyl)propane; T_t, phase transition temperature; $NaDodSO_4$, sodium dodecyl sulfate; NMR, nuclear magnetic resonance.

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is determined with a Beckman spectrophotometer Acta C III at 280 nm by using a value of $A_{1cm}^{1\%}$ of 20.1 (Kronman & Andreotti, 1964).

L-α-Dimyristoylphosphatidylcholine is obtained from Sigma. It shows a single spot on thin-layer chromatography in chloroform–methanol–acetic acid–water (50:25:7:3 v/v/v/v). It certainly contains less than 1% and probably no lysophosphatidylcholine.

1,3-Di(1-pyrenyl)propane (DPP) is synthesized by a modification of the method described by Zachariasse & Kühnle (1976). The chalcone, 1-(1-pyrenoyl)-2-(1-pyrenyl)ethylene, is prepared by stirring overnight under nitrogen 500 mL of an alkaline (0.09 M NaOH) ethanol solution, containing 10 g (41 mmol) of 1-acetylpyrene and 9.4 g (41 mmol) of 1-pyrenecarboxaldehyde. The chalcone precipitates from the reaction mixture and is recrystallized from petroleum ether. Reduction of the chalcone is done in the presence of a catalyst: dichlorotris(triphenylphosphine)ruthenium(II) (Sasson & Blum, 1971). A solution of 15 g (39 mmol) of chalcone in 150 mL of benzyl alcohol in the presence of 100 mg of catalyst is refluxed under nitrogen for 3 h. 1-(1-Pyrenoyl)-2-(1-pyrenyl)ethane precipitates upon cooling and is recrystallized from CCl_4.

The ketone (1 g) is reduced to the alkane DPP by refluxing (2 h) in diethylene glycol (20 mL) containing hydrazine (1 mL, 95%) and sodium hydroxide (0.9 g). The mixture is diluted with water (80 mL) and extracted with benzene. Purification is done by preparative thin-layer chromatography. The identity of the final product has been checked by IR analysis and NMR and mass spectrometry. A stock solution (1.5 μM) of DPP in benzene is stored at −70 °C.

Buffers. All solutions are 0.1 M NaCl and 0.01 M of the appropriate buffer. At pH 4.0 and 5.0 an acetate buffer is used, at pH 6.0 a Mes (4-morpholineethanesulfonic acid) buffer, and at pH 7.4 a Tris [tris(hydroxymethyl)aminomethane] buffer.

Preparation of Phospholipid Dispersions Labeled with DPP. A chloroform solution containing 9 mg of DMPC and a suitable amount of DPP to give a DPP to lipid molar ratio of 10^{-3} is evaporated under a stream of purified, dry nitrogen. The dry residue is taken up in 10 mL of the appropriate buffer.

(*1*) *Liposomes* (*Multilamellar Vesicles*). The mixture is stirred with a Sorvall Omni Mixer for 1 min at a temperature above T_t.

(*2*) *Vesicles* (*Small Unilamellar Vesicles*). The mixture is sonicated above T_t for 25 min in a nitrogen atmosphere with an ultrasonic disintegrator MSE 150 operated at maximum power. The sample is then centrifuged for 30 min at 25000*g* at a temperature above T_t to remove titanium dust.

Addition of α-LA to DMPC Vesicles for Steady-State Measurements. DMPC vesicles are prepared as already described in the presence of DPP at a probe to lipid molar ratio of 10^{-3}; α-LA to lipid molar ratios of 0.10 are obtained as follows: 200 μL of DPP-containing vesicles (0.9 mg of lipid/mL) are added to 1 mL of an adequate α-LA solution in the same buffer. These samples are used, starting from 37 °C, either with or without preincubation for 2 h at 23 °C. For measurements with DMPC-embedded DPP, buffer is used instead of α-LA solution.

So that the effect of the contaminants of α-LA on I_E/I_M measurements could be checked, β-lactoglobulin and serum albumin are incubated separately with DPP-containing DMPC vesicles at pH 4.0 and 23 °C. The amount used is 10% of the α-LA amount used in our experiments. No effect on I_E/I_M is observed.

Steady-State Fluorescence Measurements. Right-angle steady-state fluorescence measurements are performed with an Aminco Bowman ratio spectrofluorometer. The cell compartment is thermostated and the temperature of the cell is monitored with a thermocouple. Temperature increments are 0.5–4.0 °C, starting from 37 °C downward. Control experiments with increasing temperature show no difference. The sample is allowed to equilibrate for 10 min at each temperature before the spectra are recorded on an X-Y recorder. I_E and I_M are measured at 480 and 397 nm, respectively. The fluorescence spectra are uncorrected.

Kinetic Measurements. DMPC vesicles are prepared as already described in the presence of a suitable amount of DPP to give a probe to lipid molar ratio of 7×10^{-4}. An α-LA to lipid molar ratio of 0.17 is obtained by adding 30 μL of concentrated α-LA solution, containing 24 mg of α-LA/mL of water, to 2 mL of DMPC vesicles with a concentration of 0.1 mg of phospholipid/mL of buffer. Similar results are obtained by adding a concentrated DMPC solution to a diluted α-LA solution. The DMPC vesicles are maintained at 23 °C in the thermostated cell compartment of the spectrofluorometer. The α-LA solution is also thermostated at 23 °C before mixing with the DMPC vesicles in the cell. Analogous reference solutions are obtained by using buffer instead of an α-LA solution. Time scans of monomer and excimer emission are done on separate but identically prepared samples.

Energy Transfer Measurements. DMPC vesicles containing DPP at a relatively high probe to lipid molar ratio of 10^{-2} (in order to balance the probe vs. tryptophan fluorescence emission) and DMPC vesicles without DPP are prepared as already described. α-LA is added to DMPC vesicles either with or without DPP. In both cases α-LA to lipid molar ratios of 0.100, 0.050, 0.025, and 0.0125 are obtained as described in the section on steady-state measurements. Experiments are done at different pH values. The samples with α-LA added are allowed to preincubate for 2 h at 23 °C. In these experiments an Aminco SPF-500 spectrofluorometer coupled with a Hewlett-Packard 9815 calculator is used. This explains the different shape of the emission spectra as compared with the spectra obtained with the Aminco Bowman ratio spectrofluorometer in all other experiments. Those spectra are also uncorrected.

Results

Phase Transitions in DMPC Dispersions from Steady-State Fluorescence Measurements. As can be seen from Figure 1 the fluorescence spectrum of DPP incorporated in a DMPC dispersion shows a structured emission from 370 to 430 nm, originating from the monomer, and a structureless emission at about 480 nm, arising from the intramolecular excimer. Figure 1 shows also that the shape of the fluorescence excitation spectrum of DPP is independent of the wavelength of analysis. The excitation spectra are identical with the absorption spectrum of DPP (not shown). All these features indicate that the excimer emission originates from the excited monomer. With decreasing temperature I_E decreases concomitantly with an increase of I_M. An isoemissive point is observed at 448 nm between 10 and 37 °C. Figure 2 [(●)] shows that I_E/I_M decreases with decreasing temperature. This curve is independent of pH in the interval between 4.0 and 7.4. A discontinuity is observed in the vicinity of T_t of the bilayer.

Interaction of α-LA with DMPC Vesicles Measured by Steady-State Fluorescence. Influence of pH and Preincubation. We have studied the interaction of DMPC with α-LA at an α-LA/DMPC molar ratio of 0.10. Figure 2 shows the

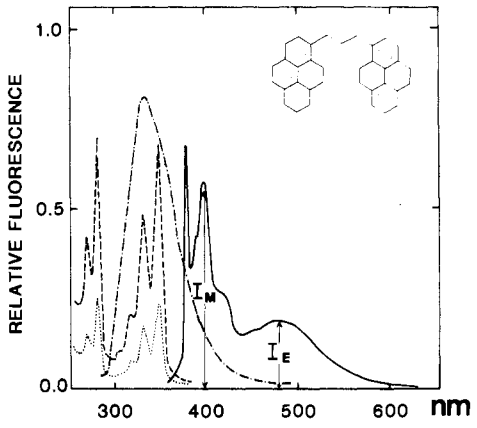

FIGURE 1: Emission and excitation spectra of DPP incorporated in DMPC vesicles at a DPP to lipid molar ratio of 10^{-3}. Temperature is 33 °C and pH 7.4: (—) emission spectrum at λ_{exc} 348 nm; (---) excitation spectrum for monomer fluorescence at λ_{em} 397 nm; (···) excitation spectrum for excimer fluorescence at λ_{em} 480 nm; (-·-) emission spectrum of α-LA added to DMPC vesicles at an α-LA to lipid molar ratio of 0.025 and λ_{exc} 283 nm.

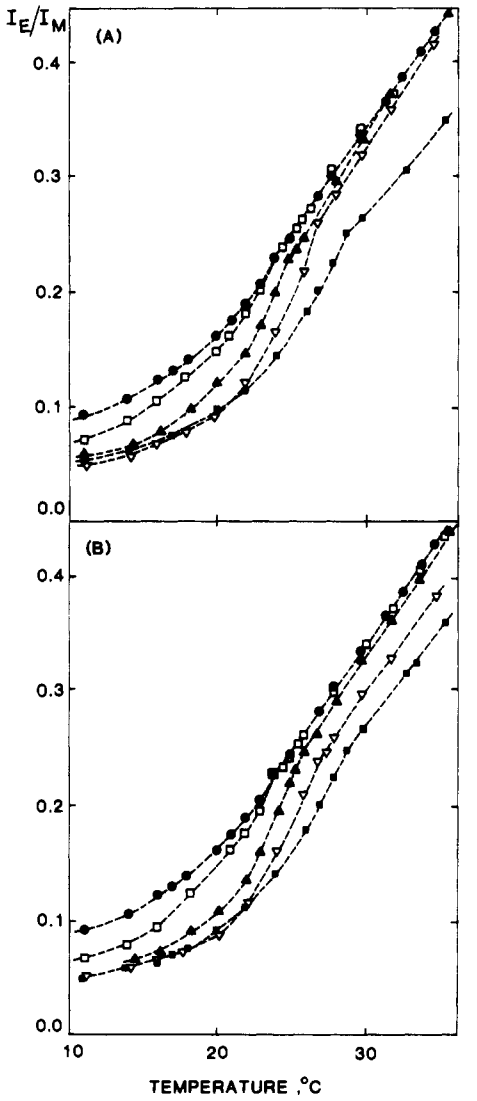

FIGURE 2: Change of the ratio I_E/I_M as a function of temperature and pH at an α-LA to DMPC molar ratio of 0.10: (■) pH 4.0, (▽) pH 5.0, (▲) pH 6.0, and (□) pH 7.4. (●) DMPC vesicles without added α-LA. (A) Without preincubation at 23 °C; (B) after 2-h preincubation at 23 °C.

influence of pH on the ratio I_E/I_M, without (Figure 2A) and with preincubation at 23 °C for 2 h (Figure 2B).

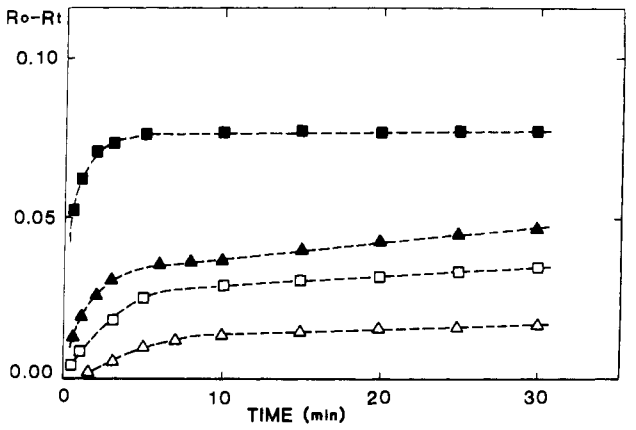

FIGURE 3: Kinetics of complex formation between α-LA and DMPC at 23 °C. $R_0 - R_t$ is the difference between the ratio I_E/I_M in DMPC vesicles (R_0) and I_E/I_M at a time interval t after adding α-LA (R_t): (■) pH 4.0, (▲) pH 5.0, (□) pH 6.0, and (△) pH 7.4.

At pH 7.4, above T_t, whether the mixture has been preincubated or not, the ratio I_E/I_M remains the same as that for pure DMPC vesicles, and T_t itself remains unaffected. Below T_t, however, we note a slight decrease of I_E/I_M. This effect is somewhat more pronounced after preincubation.

At pH 6.0 and without preincubation we observe a decrease of I_E/I_M appearing below 31 °C and a shift of T_t to a higher value (25 °C). After preincubation, however, I_E/I_M is reduced over the whole temperature range, accompanied by a shift of T_t to 26 °C.

At pH 5.0 (about the isoelectric point of α-LA) I_E/I_M is reduced, with as well as without preincubation, over the entire temperature range studied. Furthermore, in both conditions T_t is shifted to about 27 °C.

At pH 4.0 we observe a nearly equal decrease of I_E/I_M over the whole temperature range independent of preincubation. In addition T_t is shifted to 29 °C.

Kinetic Measurements. The I_E/I_M vs. T plots (Figure 2) suggest that the intensity and/or amount of complex formation of α-LA with DMPC is higher at lower pH values. This is further supported by our kinetic studies at 23 °C as a function of pH. We have measured the change of I_M at a fixed wavelength of 397 nm, after adding α-LA, as a function of time for one sample. We have also monitored the change of I_E, measured at 480 nm, for an identical second sample. R_0 is the ratio I_E/I_M in pure DMPC. R_t represents the ratio I_E/I_M at a time interval t after injecting α-LA. The difference, $R_0 - R_t$, reflects either the intensity of α-LA interaction or the amount of complex formed or both (Figure 3).

Energy Transfer Measurements. The tryptophan emission band of α-LA overlaps extensively with the absorption maxima of DPP as shown in Figure 1. Therefore we can expect, especially at pH 4.0, that resonance transfer of energy could take place (Förster, 1948) between the protein tryptophan(s) and the probe if these two species approach each other to less than the critical distance. The appearance of energy transfer would provide a supplementary indication of the penetration of α-LA into the DMPC membrane.

For each sample, the emission spectrum is recorded first at 23 °C and subsequently at 34 °C. Excitation is done at 292 nm, corresponding to a maximum in the excitation spectrum of α-LA and to a minimum in that of DPP (Figure 4).

Emission spectra (Figure 5) are recorded for the following samples: a DMPC dispersion without DPP but containing a suitable amount of α-LA (Figure 5A, curve a), a DMPC dispersion labeled with DPP and without α-LA (Figure 5B, curve c), and a DMPC dispersion labeled with DPP and with

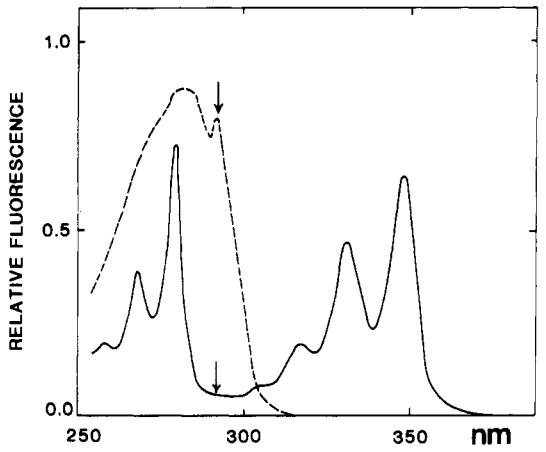

FIGURE 4: Optimal excitation wavelength for energy transfer measurements (arrows). (---) Excitation spectrum of α-LA in DMPC vesicles at λ_{em} 332 nm. The α-LA to lipid molar ratio is 0.025. (—) Excitation spectrum of DPP introduced in DMPC vesicles at λ_{em} 397 nm. The DPP to lipid molar ratio is 10^{-2}.

an appropriate amount of α-LA (Figure 5A, curve b). When α-LA is added to DMPC labeled with DPP, the tryptophan emission is quenched as shown in Figure 5A. The emission spectrum of DMPC-embedded DPP in the presence of α-LA is obtained by subtraction of the tryptophan contribution (curve a, after normalization in the 320–340-nm region) from the total emission of the mixture (curve b). This difference spectrum (Figure 5B, curve d) contains the following factors: (1) the emission of DPP in DMPC vesicles, excited at 292 nm (Figure 5B, curve c); (2) the change of the ratio I_E/I_M due to microfluidity changes caused by α-LA (as a result, the overall shape of curve d is different from that of curve c); (3) the transfer of energy. In order to obtain information about the energy transfer, we have to take account of the first two factors. Therefore, curve c (the residual DPP emission) is corrected for the "fluidity" changes induced by α-LA by giving it the shape of curve d. (Normalization is done at the isoemissive point: 448 nm.)

This manipulation yields curve e. The difference between curve d and curve e (shadowed area) is now a measure of the amount of energy transfer. An alternative measure for the amount of energy transfer is the ratio S_1/S_2 of the area under curve d (S_1) to the area under curve e (S_2).

We observe an increase of the ratio S_1/S_2 with increasing α-LA to lipid molar ratio. This is especially pronounced at 34 °C. The ratio S_1/S_2 is also influenced by pH. Indeed, measurements done at 23 °C with an α-LA to lipid molar ratio of 0.025 and at pH values of 4.0, 5.0, 6.0, and 7.4 result in S_1/S_2 values of respectively 1.91, 1.10, 1.09, and 1.01.

Discussion

In this work our main intention has been to study the applicability of the intramolecular excimer forming probe DPP to evaluate structural changes of membrane interiors, induced by lipid–protein interactions.

From the first part of our data [Figure 2, (●)], concerning the use of DPP in pure phospholipid membranes, we conclude that this intramolecular excimer forming molecule is sensitive to the temperature-induced gel to liquid-crystalline phase transitions. This confirms the results obtained previously with nonconjugated bichromophoric systems in model membranes (Dangreau et al., 1979; Georgescauld et al., 1980; Melnick et al., 1981).

In our experiments the ratio I_E/I_M increases with temperature. According to the kinetic scheme proposed by Goldenberg et al. (1978), this means that excimer fluorescence

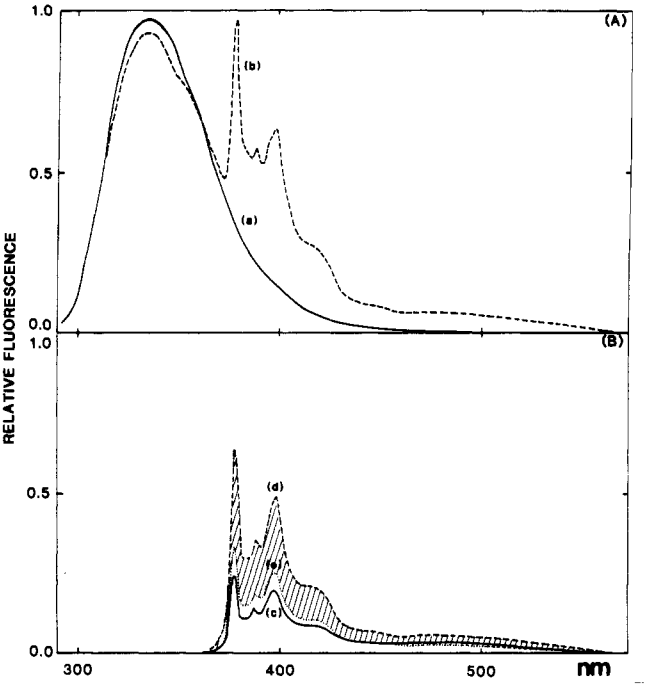

FIGURE 5: Energy transfer deduced from emission spectra. (A) (Curve a) Observed emission spectrum of α-LA in DMPC vesicles; (curve b) observed emission spectrum of DPP in DMPC vesicles containing α-LA. (B) (Curve c) observed emission spectrum of DPP incorporated in DMPC vesicles; (curve d) emission spectrum of DPP in DMPC vesicles in the presence of α-LA obtained by subtraction of the normalized tryptophan emission curve a from the emission curve b; (curve e) curve c adjusted to account for the presence of α-LA. The shadowed area is a measure for energy transfer; the α-LA to lipid molar ratio is 0.025. Preincubation is done at 23 °C; λ_{exc} 292 nm; T 23 °C; pH 4.0.

is determined by the rate of excimer formation, which is in turn dependent on the viscous resistance of the medium. This statement is only valid on the condition that excimer dissociation is not significant as, in this case, the ratio I_E/I_M should decrease, which does not occur in our case.

Of course, we are fully aware of the fact that the ratio I_E/I_M of DPP embedded in DMPC dispersions does not reflect the "viscosity" of the membrane interior, as it does in organic solvents. This problem has recently been discussed in extenso (Melnick et al., 1981). Rather, in the case of DPP the ratio I_E/I_M has to be considered as a phenomenological parameter of the membrane. Thus, the technique is suited for monitoring structural changes occurring in membranes. The interest of DPP in the study of membranes comes from its capacity to report not only on the physical state of pure phospholipid vesicles but also on protein–phospholipid complexes. An essential characteristic of this probe is that at its excitation wavelength (348 nm) there is no overlap with the protein absorption (Figure 4).

The interaction of a protein with a phospholipid dispersion is known to be dependent on its conformation and hence on the pH of the medium (Lenaz, 1977). It has been shown (Hanssens et al., 1980) that α-LA behaves differently depending on pH. Microcalorimetry has shown for the enthalpy of interaction a maximum at pH 4 and 5 and situated around the phospholipid phase transition temperature. This is accompanied by the formation of stable protein–phospholipid complexes (molar ratio around 1:80) that are distinctly smaller than the original vesicles, as shown by gel filtration and electron microscopy. Measurements on the effect of salt addition have led to the conclusion that, at neutral pH, α-LA merely adsorbs to the vesicle outer surface by electrostatic

interaction, whereas at more acidic pH (4 and 5) the interaction has a hydrophobic nature. These observations have been corroborated by tryptophan fluorescence studies and diphenylhexatriene fluorescence polarization measurements (Herreman et al., 1981a). The former shows that, upon complexing, α-LA is shielded from the solvent, whereas from the latter it follows that, at pH 4, α-LA strongly disturbs the phospholipid phase at all temperatures. In contrast, at pH 5 penetration only occurs around T_t. This only confirms previous studies on monolayers (Hanssens & Van Cauwelaert, 1978). The perturbation seems to depend on a pH-induced conformational change that is also affected by preincubation at T_t as depicted in Figure 2. At pH 5.0 the reduction in I_E/I_M above T_t (27 °C) is more pronounced after preincubation, suggesting that the interaction of α-LA with DMPC is a time-dependent process. At pH 4.0 it seems that the conformational change of α-LA favors a strong and rapid interaction with the lipid bilayer.

This is further supported by our kinetic studies as a function of pH. Indeed, the rate of complex formation, as indicated by the slope of the curves in Figure 3, increases from neutrality to acidity. Except at pH 4.0, the interaction between α-LA and the phospholipid seems to follow a two-step mechanism: a fast step completed after about 5 min, followed by a slow one. On the assumption that isothermic changes in I_E/I_M are an indication of the amount of interacting protein or of the intensity of interaction, it follows that α-LA interaction at pH 5.0, 6.0, and 7.4, in contrast to pH 4.0, is relatively small.

The major evidence, however, for α-LA penetrating the bilayer comes from energy transfer. Our experiments demonstrate that there is indeed energy transfer at pH 4.0 from one or more of the four tryptophan residues of α-LA to membrane-buried DPP. Independent gel filtration experiments (not illustrated) have shown that at pH 4.0 DPP does not detectably bind to α-LA in the absence of DMPC.

From the difference in the emission spectra of DPP in DMPC vesicles, with and without α-LA added, no noticeable energy transfer at pH values 5.0, 6.0, and 7.4 could be deduced. In contrast, energy transfer is manifested at pH 4.0, indicating a close approach between α-LA and DPP. The increased transfer efficiency in the presence of increasing amounts of α-LA observed at 34 °C and at pH 4.0 may originate from either an enhanced mobility of DPP and/or α-LA in the liquid hydrocarbon layer, allowing a closer approach, or alternatively a larger amount of interacting α-LA.

Our energy transfer measurements support the earlier conclusions that α-LA acts as an intrinsic protein at acidic pH, strongly disturbing the membrane structure. Analogous results are reported by Herreman et al. (1981b) using 1,6-diphenyl-1,3,5-hexatriene as a probe.

Conclusions

Our experiments clearly demonstrate the applicability of intramolecular excimer forming systems, especially DPP, in the study of protein–phospholipid interactions at least in our model system. The major effect leading to α-LA–lipid interactions is probably related to conformation: either conformation of the phospholipid phase, which is extremely favorable around T_t, or conformation of the protein induced by pH changes. The latter alternative seems to predominate as evident from our steady-state, kinetic, and energy transfer studies. These measurements agree with observations from microcalorimetric, gel chromatographic, and fluorescence polarization studies done in our laboratory.

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