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Protein-dependent Reduction of the Pyrene Excimer Formation in Membranes

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Abstract. The presence of proteins in lipid bilayers always decreases the excimer formation rate of pyrene and pyrene lipid analogues in a way that is related to the protein-to-lipid ratio. Energy transfer measurements from intrinsic tryptophans to pyrene have shown (Engelke et al., 1994), that in microsomal membranes, the excimer formation rate of pyrene and pyrene fatty acids is heterogeneous within the membrane plane, because a lipid layer of reduced fluidity surrounds the microsomal proteins. This study investigates whether of not liposomes prepared from egg yolk phosphatidylcholine with incorporated gramicidin A give results comparable to those from microsomal membranes. The results indicate that the influence of proteins on the lipid bilayer cannot be described by one unique mechanism: Small proteins such as gramicidin A obviously reduce the excimer formation rate by occupying neighboring positions of the fluorescent probe and thus decrease the pyrene collision frequency homogeneously in the whole membrane plane, while larger proteins are surrounded by a lipid boundary layer of lower fluidity than the bulk lipid.

The analysis of the time-resolved tryptophan fluorescence of gramicidin A incorporated liposomes reveals, that the tryptophan quenching by pyrene is stronger for tryptophans located closely below the phospholipid headgroup region because of the pyrene enrichment in this area of the lipid bilayer.

Key words: Energy transfer — Intrinsic tryptophans — Pyrene excimer formation — Membrane fluidity — Gramicidin incorporated liposomes — Microsomal membranes

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Introduction

Biological membranes consist of a bilayer of different classes of lipids, into which several types of integral proteins are integrated. The fractional membrane area which is occupied by proteins (determined from the dry weight fraction and the molecular size) varies in the range from 20 to 75%. Fluorescence spectroscopic measurements have shown, that due to their greater mass not only the lateral mobility of the proteins is lower than that of the lipids (Vaz et al., 1984), but also the lateral mobility of the lipids is reduced in the presence of integral proteins compared to pure phospholipid bilayers.

One approach to the study of lateral lipid diffusion is based on the excimer formation of pyrene or pyrene-labeled fatty acids and phospholipids (Galla & Sackmann, 1974; Galla et al., 1979). In solution, the excimer formation depends on the local concentration, the temperature and the viscosity of the solvent (Birks, 1970; Förster, 1969). Galla and Sackmann (1974) showed that the excimer formation kinetics of pyrene in lipid bilayers depends on its diffusion in the bilayer plane. Variations in the lipid bilayer fluidity can be characterized by the intensity ratios of the excimer and monomer fluorescence (Galla & Sackmann, 1974; Vanderkooi & Callis, 1974).

The presence of proteins in biological membranes has important consequences for the in-plane diffusion. This effect on the long-range diffusion of lipids has been evaluated by several authors (Eisinger et al., 1986; Baros et al., 1991). The presence of proteins also decreases the ratio of excimer-to-monomer fluorescence (E/M) in a way which is related to the protein-to-lipid ratio (Almeida et al., 1982). According to the Milling Crowd Simulation Model from Eisinger et al. (1986) proteins are considered as lipid-forbidden hexagons in the bilayer. Four different effects may contribute to a protein-

induced reduction of the excimer formation of pyrene and pyrene lipid analogues: (i) Proteins represent mechanical barriers for lipids and fluorescent probes. Proteins statistically reduce the excimer formation by occupying neighboring positions of the probe and increase the path length of the pyrene molecules before they collide. (ii) Lipids adjacent to the protein surface are immobilized and their exchange frequency (and that of the pyrene molecules in this area) may be significantly lower than in the bulk lipid matrix. (iii) The rotational freedom of the lipid acyl chains adjacent to the proteins is reduced due to a protein lipid interaction. (iv) Molecules such as pyrene may be motionally restricted at the protein lipid interface due to charge transfer interaction between aromatic groups of the molecule and the protein (Engelke et al., 1995), or pyrene molecules are bound at hydrophobic protein pockets (Jones & Lee, 1985; Imai, 1982).

We have shown (Engelke et al., 1994), that in microsomal membranes a lipid layer of reduced fluidity surrounds the proteins. Pyrene and pyrene fatty acids do not diffuse homogeneously within the membrane plane, because a fluidity gradient exists from the lipid bilayer to the proteins.

A broad spectral overlap exists from the emission spectrum of intrinsic tryptophans (donor) and from the absorption spectrum of pyrene and pyrene derived probes (acceptor). By nonradiative energy transfer from intrinsic tryptophans only the pyrene molecules in the protein surrounding (approximately the Förster radius) are selectively excited, the excimer formation rate in the near protein environment can be separated from that in the bulk lipid layer.

It was the aim of this study to investigate, whether liposomes doped with polypeptides may be regarded as models for natural membranes. As natural membranes we used microsomal membranes prepared from the endoplasmatic reticulum of pig livers (PLM), because these membranes are well characterized in their structural and functional aspects (Schulze & Staudinger, 1975).

We have used gramicidin D incorporated into egg yolk phosphatidylcholine liposomes (GAL) as a model system. Simulations using the Milling Crowd Model (Eisinger et al., 1986) have shown that the diffusion coefficient of lipids is reduced to a much greater extent by small proteins than by larger ones, when they occupy the same fractional area. Gramicidin D is a natural mixture that contains predominantly (87%) gramicidin A. Gramicidin A is a well described (Woolley & Wallace, 1992) peptide of 1867 Dalton. This polypeptide has the ability to form conducting cation-selective transmembrane channels, and to monitor its effect on lipid organization it is necessary to study lipid-protein interaction. Because of its hydrophobic nature gramicidin A incorporates easily into the acyl chain region of phospholipid bilayers, where it can exist in different conformations depending on the organic solvent used for the membrane preparation procedure (Killian & Urry, 1988; Bañó et al., 1991). We have used trifluoroethanol as the solvent to get the so-called channel conformation of gramicidin (Tournois et al., 1987), a single-stranded β-helical membrane spanning dimer of about 25–30 Å in length, consisting of two gramicidin molecules. Gramicidin A contains 4 tryptophan residues which are all localized in the proximity of the C-terminus (Woolley & Wallace, 1992). Fluorescence lifetime measurements of gramicidin A exhibit a double exponential character consisting of a very short-lifetime component (~1ns) and a longer one (~3ns), each with a similar contribution to the total fluorescence intensity (*this paper*).

In this paper we have compared the E/M ratio in microsomes (PLM) and gramicidin A containing liposomes (GAL) at direct excitation at 336 nm or at excitation by energy transfer from intrinsic tryptophans. Moreover, we determined the transversal pyrene localization in GAL and PLM by studying the fluorescence quenching of intrinsic tryptophan by pyrene. A consistent interpretation leads to the suggestion that different mechanisms are responsible for the reduced fluidity in membranes containing small (GAL) or large (PLM) proteins.

Materials and Methods

Preparation of Liposomes and Microsomes

Liposomes were prepared according to Bañó et al. (1991) to achieve a gramicidin dimer as the major gramicidin conformation in the lipid bilayer. Egg yolk phosphatidylcholine (EPC) (Sigma, Deisenhofen, Germany) was dissolved in dichloromethane/methanol (2:1) and evaporated to deposit a lipid film on the wall of a glass tube. Gramicidin D (Sigma) dissolved in trifluoroethanol was added and also evaporated. For control measurements, liposomes without gramicidin were prepared. Final traces of the residual solvent were removed under vacuum overnight. The lipid-gramicidin mixtures were dissolved in an appropriate amount of TRIS buffer solution (100 mM TRIS, pH 7.4). The samples were then vigorously vortexed at room temperature, followed by sonication for 15 min at 50°C by using an ultrasonic generator (Bandelin Elektronik, Berlin, Germany) at maximum power settings and 30% cycle. In the process of preparation we systematically degassed the probes with nitrogen to avoid lipid oxidation. After sonication, the samples were centrifuged for 5 min at 4,000 g to remove heavier particles and remaining multilamellar aggregates from the preparation. The lipid content in the liposomes was determined according to Stewart (1980) and the gramicidin A concentration was evaluated by measuring the absorbance at 280 nm after dilution of the sample in methanol using a molar extinction coefficient of 20700 cm^{-1} M^{-1} (Bañó et al., 1991). The final gramicidin concentration was 0.94 mg/ml. The gramicidin-to-lipid mole ratio of our preparations was 0.08.

Pig livers were supported from the public slaughterhouse. Microsomal fractions were prepared by differential centrifugation (Lu, 1976). Microsomes were suspended in a 0.01 M potassium phosphate buffer, pH 7.7, with 20% glycerol, and stored at 213 K. Microsomal

protein was determined by the method of Peterson (1977). All measurements were carried out on material taken from the same preparation. The molar protein to lipid ratio was about 0.025.

Steady-State Fluorescence Measurements

The steady-state sample fluorescence was recorded upon front face excitation using the equipment built in our laboratory and described previously (Kawski & Nowaczyk, 1990; Kawski et al., 1994). In all measurements we used a 1 mm cuvette to keep the optical density below 0.1 and to avoid scattering and secondary effects. For direct measurements, the fluorescence of pyrene (high purity; Molecular Probes, Oregon) was excited at 336 nm. Fluorescence spectra were scanned from 360 to 500 nm. In the case of energy transfer measurements, the excitation wavelength was 292 nm (high tryptophan but low pyrene absorption) and the spectra were recorded from 300 to 500 nm. For the calculation of the excimer to monomer fluorescence ratio we used the signal intensities at 393 nm (peak of the pyrene monomer band) and at 468 nm (maximum of the pyrene excimer band). Pyrene was added from a 10^{-3}-M stock solution in ethanol to the liposomal or microsomal suspension to give a final concentration of 0.5–3 mol% pyrene per lipid.

Energy-transfer efficiencies (ET) were determined from the quenching of the tryptophan fluorescence according to the equation:

$$ET = 1 - I/I_o \tag{1}$$

where I and I_o are the tryptophan emission intensities in the presence and absence of pyrene probes, respectively. The intensities were evaluated from the peak height of the 330 nm tryptophan fluorescence under excitation at 292 nm.

According to Kleinfeld and Lukacovic (1985) the pyrene fluorescence due to energy transfer from tryptophan is given by:

$$I = I_{\mathrm{ET}} + I_D + I_{\mathrm{Trp}} + I_R \tag{2}$$

in which I_{ET} is due to the nonradiative energy transfer, I_D is due to direct emission, I_{Trp} is due to the tryptophan emission background and I_R due to radiative migration. The influence of these non-Förster mechanisms on the evaluation of the pyrene monomer and excimer fluorescence have been described (Engelke et al., 1994).

Time-resolved Fluorescence Measurements

Fluorescence lifetimes were extracted from the fluorescence decays using bi-exponential analysis. Fluorescence decays were measured using the standard time-correlated single photon-counting method. Samples were excited at 292 nm by light pulses with a time halfwidth of $t_{1/2} < 5$ ps at a frequency of 700 kHz. As a light source we used the second harmonic of a R6G dye laser equipped with the cavity dumper, pumped by a mode-locked Coherent Antares 76 YAG laser system. As detectors served the Antel ARS-2 fast photodiode in start channel and a Philips XP 2020 Q photomultiplier in stop channel. In the electronic part we applied a constant fraction discriminator TC 454 Tennelec, a TC 864 Tennelec time-amplitude converter and Nucleus-Oxford Personal Computer Analyser PCA II 8000. For deconvolution a commercial software Edinburgh Analytical Instruments FLA-900 was employed. We checked shape and location of the tryptophan part of the fluorescence spectra independently and virtually no change in these characteristics was found at concentrations $\leqslant$3 mol%.

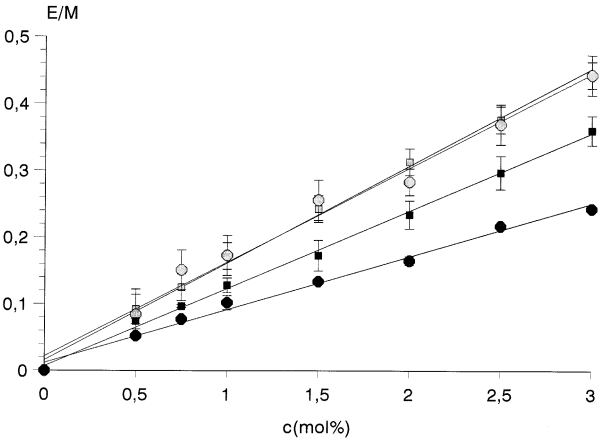

Fig. 1. Excimer-to-monomer fluorescence ratio (E/M) for microsomes and liposomes. Symbols denote experimental E/M values for microsomes excited at 292 nm (●) and 336 nm (■) as well as for liposomes excited at 292 nm (○) and 336 nm (□), respectively.

Results

Excimer-to-Monomer Fluorescence Ratio

In Fig. 1 the intensity ratio of the excimer-to-monomer emission (E/M) for PLM and GAL is plotted against the probe concentration, both for direct excitation at 336 nm and for excitation by energy transfer from intrinsic tryptophans. At the excitation wavelength of 336 nm all pyrene molecules in the bilayer are excited with the same probability, but when excited at 292 nm only the pyrene molecules in the immediate protein surrounding can be excited (approximately the Förster radius). The fluorescence intensity of pyrene for excitation by energy transfer was evaluated according to the protocol given in Materials and Methods (Eq. 2). The E/M ratio increases linearly with increasing pyrene concentration for all samples. While for GAL the E/M increase is the same for direct and indirect excitation, pyrene excitation by energy transfer from microsomal tryptophan always results in a lower E/M ratio due to a reduced excimer formation rate compared to direct excitation.

Tryptophan Quenching

Increasing pyrene concentration in the lipid bilayer results in a linear decrease of the microsomal and gramicidin tryptophan fluorescence due to energy transfer from excited tryptophans to pyrene. Figure 2 shows the Stern-Volmer plot of the tryptophan quenching for PLM and GAL up to a pyrene-to-lipid ratio of 3 mol%. In GAL, the tryptophan quenching by pyrene is much stronger than in PLM. The different extent of tryptophan quenching in both membrane preparations is confirmed

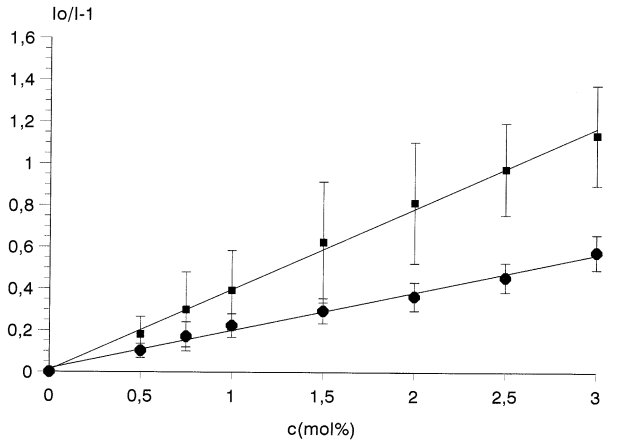

Fig. 2. Stern-Volmer plot of tryptophan fluorescence quenching by pyrene in microsomes (●) and in gramicidin A incorporated liposomes (■).

by the time-resolved determination of the tryptophan fluorescence decay times. Tryptophan lifetime measurements (Table) reveal two components, both, in proteins from microsomes and in gramicidin A. In correspondence with the steady-state measurements, tryptophan lifetime decreases linearly with increasing pyrene concentration due to the collisional quenching. The decrease of the shorter lifetime component with increasing pyrene concentration is stronger than that of the long lifetime component, especially in gramicidin A. It should be stressed that the parameters obtained within the two-exponential model although not perfect are consistent, which suggests that such analysis may be used for qualitative analysis at low and moderate acceptor concentrations. For deviations from this model at highest concentrations of pyrene, the increasing strength of energy transfer is responsible.

Energy Transfer from Intrinsic Tryptophan to Pyrene

The tryptophan fluorescence quenching and the decreased tryptophan fluorescence lifetimes caused by pyrene indicate a more efficient nonradiative energy transfer from tryptophan to pyrene in GAL than in PLM. Energy transfer efficiency data for different molar pyrene to lipid ratios are plotted in Fig. 3.

Discussion

For pig liver microsomes (PLM) as well as for liposome incorporated gramicidin (GAL) the evaluation of the excimer to monomer ratio (E/M ratio) reveals a linear increase with increasing molar pyrene concentration in the bilayer for both, direct excitation at 336 nm and at excitation by energy transfer (Fig. 1). There are two great differences between the results obtained from PLM and GAL: (i) In spite of the high molar concentration of incorporated gramicidin A (8% compared to the molar protein to lipid ratio of 2.3% in PLM) the overall E/M ratio is higher in GAL than in PLM. (ii) A difference in the mean excimer formation rate compared to the excimer formation rate in the protein surrounding upon energy transfer excitation from tryptophan was observed for PLM but not for GAL.

Table. Lifetimes of tryptophans in (A) microsomes and (B) gramicidin A incorporated liposomes as a function of increasing pyrene concentrations in the bilayer

A. Microsomes c[mol%]	τ_1[ns]	τ_2[ns]	α_1[%]	α_2[%]	$\Delta\tau_1$[ns]	$\Delta\tau_2$[ns]	χ^2
0.00	3.78	1.25	47.1	52.9	0.022	0.016	1.44
0.50	3.75	1.22	47.2	52.8	0.017	0.017	1.55
0.75	3.74	1.22	47.4	52.6	0.018	0.015	1.66
1.00	3.67	1.15	47.9	52.1	0.018	0.017	1.68
1.50	3.58	1.11	48.3	51.7	0.017	0.018	1.72
2.00	3.55	1.07	48.9	51.1	0.015	0.014	1.77
2.50	3.49	1.06	49.2	50.8	0.017	0.015	1.89
3.00	3.47	1.02	49.9	50.1	0.018	0.014	2.05
B. Gramicidin A							
0.00	3.38	1.10	40.1	59.9	0.020	0.014	1.24
0.50	3.35	1.07	40.0	60.0	0.015	0.015	1.25
0.75	3.34	1.02	39.5	60.5	0.017	0.013	1.32
1.00	3.27	0.95	39.1	60.9	0.019	0.016	1.37
1.50	3.18	0.91	39.2	60.8	0.022	0.014	1.42
2.00	3.15	0.87	38.3	61.7	0.016	0.015	1.51
2.50	3.07	0.83	37.5	62.5	0.019	0.013	1.72
3.00	2.99	0.77	37.0	63.0	0.017	0.015	1.92

τ[ns] lifetimes of the two components
α[%] fractional contribution of the two lifetime components
$\Delta\tau$[ns] standard deviations
χ^2 goodness of fit

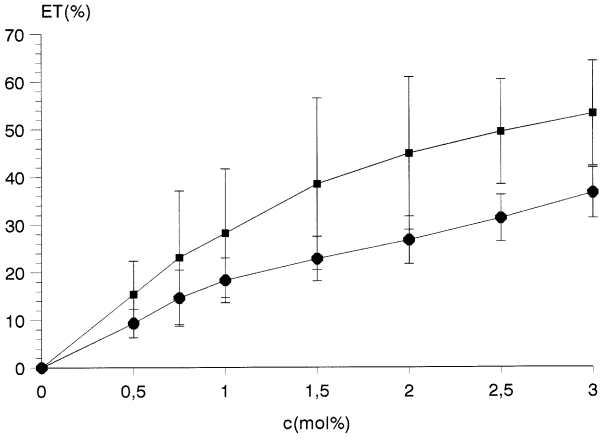

Fig. 3. Energy transfer efficiency from intrinsic tryptophans to pyrene in microsomes (●) and gramicidin A incorporated liposomes (■).

To explain the difference in the mean E/M ratio between PLM and GAL, we have calculated the fractional area covered by proteins assuming an average protein diameter of 30 Å for microsomal membranes and 15 Å (Wu et al., 1978) for gramicidin A. The fractional area f is defined by the sum of obstacles, i.e., proteins (Eisinger et al., 1986), divided by the total area of the membrane. Since the higher molar gramicidin concentration compensates for the smaller diameter of the polypeptide, the fractional protein areas of both preparations do only differ a little: $f = 0.20$ for GAL and 0.23 for PLM. According to Saxton's modified percolation theory (1982), a semiquantitative cubic function for the diffusion coefficient governs the dependence of the diffusion rate on the fractional area. Small changes in the fractional area lead to large changes in the diffusion coefficient as we observe in our measurements.

Our results can also be discussed by the depolarization studies on gramicidin A incorporated DOPC vesicle bilayers (Muller et al., 1995). Their measurements reveal little change in the steady state fluorescence anisotropy in the hydrophobic bilayer center, but a much larger effect on the outer bilayer region, when gramicidin A is in its channel conformation. The ordering effect of gramicidin A on the bilayer is found to depend both on the conformation of the polypeptide and the depth of the bilayer at which the order is probed. Since in our GAL preparation, gramicidin A is supposed to be in its channel confirmation and pyrene mainly labels the acyl chain region of the bilayer, only small effects on the pyrene excimer formation are reasonable.

On the other hand, our measurements are contradictory to the results from Eisinger et al. (1986): Milling Crowd Model Simulations of pyrene lipids reveal that the diffusion coefficient depends both on the fractional area covered by the obstacles and on their size. They propose from their calculation, that the lipid diffusion is reduced to a much greater extent by small obstructions than by larger ones at the same fractional area.

A further difference between the results from GAL and PLM (Fig. 1) is that the increase of the E/M ratio is stronger at direct excitation than at excitation by energy transfer for PLM but not for GAL. While the E/M ratio at direct pyrene excitation represents the mean value of the overall excimer formation, E/M ratios at excitation by energy transfer only result from pyrene molecules in the neighborhood of the proteins. Figure 1 shows that the excimer formation in the vicinity of the microsomal proteins is much more affected than in the bulk lipid region. As described in the introduction, a decrease of excimer formation by proteins originates from either statistical factors (increased path length and decreased pyrene collision frequency) and/or from differences in local order and dynamics of lipids in the vicinity of proteins. In microsomal membranes, the excimer formation near proteins is reduced compared to the average excimer formation rate, which seems in favor of the second model. Proteins dispersed in the microsomal membrane not only exert an effect on long-range diffusion like immobile obstacles, but rather affect the order of the adjacent lipid acyl chains. This model is supported by several authors (Chapman, 1982; Jost et al., 1973; Almeida et al., 1982), who propose a boundary lipid layer of reduced fluidity around proteins. The physicochemical origin of such a 'lipid boundary layer' can be explained by the 'matrass-model' introduced by Mouritsen and Bloom (1984), which is based on mechanical protein-lipid interaction, or by hydrogen bondings between indole rings of the proteins and the carbonyl groups of the adjacent lipids at the protein-lipid interface (Muller et al., 1995; Mukherjee & Chattopadhyay, 1994).

In contrast to the results obtained from PLM no difference was found in the excimer formation rate in the vicinity of gramicidin A and the bulk lipids. The reason for the different results revealed from PLM and GAL might be the heterogeneous lipid composition of the membrane of PLM. The microsomal lipid is composed of 55% PC, 20% PE, 9% PI, 7% PS and 9% others (Schulze & Staudinger, 1975). According to Pap et al. (1995) the excimer formation of dipyrenyl-labeled PI is significantly influenced by Band 3 protein, while dipyrenyl-labeled-PC is hardly affected by the presence of this protein. Nonuniform distribution of phospholipids and their interaction with membrane proteins may be rate limiting for the excimer formation in PLM. Since the lipid fraction of GAL consists of EPC only, no such reduction in the excimer formation rate in the protein surrounding could be observed. The effect of gramicidin A on the lipid morphology depends critically on the specific lipids involved (Woolley & Wallace, 1992; Turnois et al., 1987).

One also has to consider the size of the proteins. Gramicidin A is small compared to microsomal proteins. At temperatures above the phase transition, all gramicidins are randomly distributed in the lipid bilayer (Chapman et al., 1977), and each gramicidin molecule is enclosed by approximately 6 lipid molecules. At a molar protein-to-lipid ratio of 0.08 in our preparation (*see* Materials and Methods) we assume that no large lipid domains exist and probably all pyrenes may be excited by energy transfer from tryptophan. In microsomal membranes at an identical protein to lipid ratio some of the pyrene molecules may accumulate in lipid clusters. These pyrene molecules are not in Förster distance to proteins.

Fluorescence quenching (Fig. 2) and energy transfer efficiency (Fig. 3) of intrinsic tryptophans in the presence of pyrene are stronger in GAL than in microsomal membranes. For both microsomal and gramicidin tryptophan, the fluorescence is quenched linearly with in-

creasing pyrene concentration in the bilayer (Fig. 2) and modified Stern-Volmer plots reveal that all tryptophans are accessible to the quencher (*data not shown*). Fluorescence maxima for both microsomal and gramicidin tryptophans are at 330 nm. This blue shift is interpreted as being due to the shielding of the tryptophan residue from the aqueous phase either by the protein or the lipid environment. The fluorescence quenching of microsomal tryptophan is weak compared to tryptophan quenching in gramicidin, because microsomal proteins are much larger than gramicidin, which consists of only 15 amino acids. Most of the tryptophans from microsomal proteins are supposed to be deeply buried in the protein center and statistically less accessible to the acceptor molecules.

The steady-state measurements of the fluorescence quenching of tryptophan by pyrene are confirmed by the tryptophan lifetime measurements. Fluorescence lifetime analysis shows a double exponential decay, corresponding to a short- and a long-lifetime component (Table), for PLM and for GAL. The two lifetime components are ascribed to distinct tryptophan environments in the membrane. For gramicidin A it has been proposed by Mukherjee and Chattopadhyay (1994), that the short lifetime component corresponds to the self-quenching of an interacting tryptophan pair. For gramicidin A, this pair could be tryptophans-9 and -15, because of their close proximity in a β-helical configuration. The localization of the tryptophan pair responsible for the short lifetime component is near the phospholipid headgroup region at the lipid-water interface just as one of the tryptophans emitting the long-lifetime component. Only one tryptophan is localized in the hydrophobic acyl chain region near the bilayer center (Mukherjee & Chattopadhyay, 1994). Since the protein composition of the microsomal membrane is rather complex it is impossible to relate the fluorescence lifetime distribution to the tryptophan localization.

Both fluorescence decay components of the bimodal distribution decrease with increasing pyrene concentration, both, for microsomal and gramicidin tryptophan (Table), in correspondence to the steady-state quenching data (Fig. 2). Since for gramicidin A the decrease of fluorescence lifetime is stronger for the short component, we conclude, that an enrichment of pyrene occurs to be in the region of the acyl chains tightly below the phospholipid headgroups.

In summary, our results demonstrate that no unique mechanism can describe the influence of proteins on their lipid environment. Small proteins such as gramicidin A statistically reduce the pyrene excimer formation by occupying neighboring positions of the probe and thus decrease the pyrene collision frequency in the bilayer, while large proteins in microsomal membranes are surrounded by a lipid boundary layer of lower fluidity than the bulk lipid.

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