INSERTION OF PROTEINS IN THE LIPID BILAYER OF LIPOSOMES REVEALED BY FRET

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Abstract. Many processes at the level of biological membranes involve the participation of integral and/or adsorbed proteins. The proper functioning of the membrane proteins is related to the local molecular environment, one of the most important factors being the lipid bilayer. In this paper we present a method to evidence the interaction of proteins with the lipid bilayer in artificial lipid membranes using the fluorescence resonance energy transfer (FRET) method. The donor in the energy transfer process is the tryptophan residue from the proteins and the acceptor is diphenylhexatriene, a lipid bilayer fluorescencent marker. Liposomes prepared from lecitine and synthetic lipids were used as model membranes. The FRET procedure was first tested on gramicidin A inserted in the liposome bilayer and afterwards applied to study the insertion of a bacterial outer membrane porin, namely OmpF. Recording the spectra of liposomes-protein-diphenylhexatriene complex for different concentrations of the acceptor, the energy transfer was evidenced and the results were discussed in terms of FRET efficiency.

Key words: protein insertion, liposomes, fret, gramicidin, OmpF.

INTRODUCTION

The integral proteins are very important components of biological membranes assuring various functions as molecular recognition, signal transduction, ion and small molecules transport, etc. The structural complexity of the natural membrane and the large variety of processes evolving simultaneously at this level make the understanding of the mechanism of protein function in the membrane very difficult. A method to study easily the protein function is to reconstitute the protein in a model membrane. The most used model membranes are planar lipid membrane (BLM) and lipid vesicles (liposomes). Both of these variants are extensively used to study the proteins function with respect to external environment (ionic composition, pH, specific ligands, voltage across the membrane, etc.). This method is very useful in an important issue regarding the protein-lipid bilayer interactions. The new hypothesis of rafts in membranes reinforced this type of studies.

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Considered to be functional lipids-proteins microdomains (size of few nm) in natural membranes, rafts are very often related to specific lipid composition of the bilayer [2]

The first step to analyze the protein functioning in a membrane of a given lipid composition is to prove the insertion of the protein in the membrane core. The aim of our study is to give evidence for the insertion of a bacterial outer membrane porin (OmpF) in the lipid bilayer of liposomes. The OmpF is a major porin transporting ions and small molecules (up to 600 Daltons) across the outer membrane of gram negative bacteria, as *Escherichia coli* [3]. Being a channel, most of the functional studies on this protein were done by electrophysiological methods (voltage clamp on OmpF reconstituted in planar lipid bilayers). Fewer studies were devoted to the protein-lipids interaction in the membrane. In our study we used fluorescence techniques to reveal the insertion of the peptides (particularly the OmpF) into the lipid bilayer of liposomes. The most of proteins contain in their structures aromatic amino acids residues which have well known fluorescence properties. The most important among them is tryptophane (Trp) absorbing at 290 nm and emitting usually at 348 nm. But the tryptophan emission maximum may strongly shift toward lower wavelengths, as a function of the physical properties of the environment [4].

Discovered by Foerster more than 50 years ago [4], FRET (fluorescence resonant energy transfer) became one of the powerful biophysical methods to characterize the interactions between molecules located at small distances ~ 2 – 8 nm [4]. In molecular biology FRET is very often used to analyze the spatial structures of macromolecules (as protein folding, DNA packaging, etc.), supra-molecular structures (as ligand receptor interactions, etc.) [4]. FRET is used very successfully in analyzing the interactions among molecular membrane components.

In this paper we report results concerning the insertion of OmpF into the liposomes membrane, using the FRET method. The donor in these FRET experiments is Trp (as residue from proteins) and the acceptor is diphenylhexatriene (DPH), a very hydrophobic fluorescencent molecule used in fluorescence studies on membranes. In the first part of our study the FRET procedure was verified, using Gramicidin A (GA), a hydrophobic peptide which has in this composition four Trp residues [9] as donor. GA is very easily inserting in the lipid bilayer of membranes. The same procedure was afterwards used to prove the insertion of OmpF in the liposome membrane.

MATERIALS AND METHODS

The liposomes were prepared from egg lecithin and diphytanoylphosphatidylcholine (DPhPC) from Avanti Polar (USA), the gramicidin A and TMA-DPH was provided by Sigma Chemicals (Germany) and the OmpF was a gift of Dr. Mathias Winterhalter from the University of Bremen.

The liposomes were prepared following a usual method based on sonication [8]. Briefly, the lipids (lecithin or DPhPC) were dissolved in chloroform at a concentration of 10 mg/ml as a stock solution and preserved at –20ºC. 225 μl from this solution were mixed with 775 μl of a mixture of methanol-chloroform (1/1, v/v) in a very clean glass tube with round bottom and the solvent was removed by drying under nitrogen flow. The lipidic film was hydrated with 3 ml of phosphate buffer saline (PBS, 10 mM) at pH 7.4. After hydration the solution was vigorously vortexed obtaining in this way a multilamellar vesicles suspension. This suspension was sonicated in an ultrasonic bath (80 W) until clarity (~ 30 min) resulting a suspension of small unilamellar vesicles (SUV) which were used in the experiments after a dilution until a final volume of 45 ml (concentration of lipids ~ 77 μM).

The GA was dissolved in ethanol at a concentration of 5 mg/ml. Small aliquots (few microliters) of this stock solution were added to the SUV suspension to reach appropriate concentration values. The OmpF was received from Dr. Winterhalter as a solution of 1 mg/ml in PBS with 1% detergent (Octyl-POE). As for GA, few microliters of this solution were added to SUV suspension during the experiments.

The fluorescence spectra were recorded with a Floromax 3 (Horiba Jobin Yvon) fluorimeter, λ_{ex} = 290 nm and λ_{em}: 300–500 nm. The spectra were corrected for the spectral sensitivity of the emission channel of the fluorimeter.

In order to quantitatively characterize the FRET four types of spectra were recorded: the spectrum of liposomes, the spectrum of liposomes doped with protein (GA or OmpF), the spectrum of liposomes doped with TMA-DPH and the spectrum of liposomes doped with protein and TMA-DPH. The first two spectra were used to correct the third and fourth spectrum, respectively for the liposomes fluorescence and for acceptor fluorescence in the absence of the donor. The corrected spectra were used to compute the FRET efficiency with the formula:

$$E = 1 - \frac{I_n}{I_0} \tag{1}$$

where I_n is the maximal intensity of the donor emission in the presence of the acceptor (n counting the acceptor concentration value) and I_0 the maximal intensity of the donor emission in the absence of the acceptor.

RESULTS

In the first part of the study we verified the FRET procedure with GA peptide as donor. GA inserts easily in the lipid bilayer and, being shorter than one monolayer thickness is located in a close contact with the hydrophobic core of the membrane. Consequently, GA molecules are near TMA-DPH molecules. In such conditions we expect a significant energy transfer to occur. In Figure 1 the absorption and emission spectra (each of them normalized to the highest value) of Trp, GA and

DPH are plotted and the high overlapping area of GA's emission spectra and DPH's absorption one can be easily observed. In this way we prove that the main important condition for an energy transfer to occur is fulfilled by this pair of molecules.

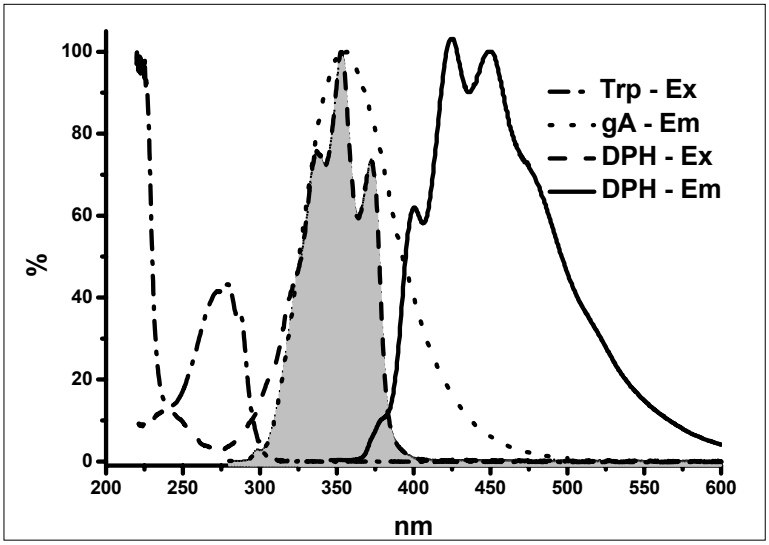

Fig. 1. Normalized absorption and emission spectra of Trp, GA and DPH.

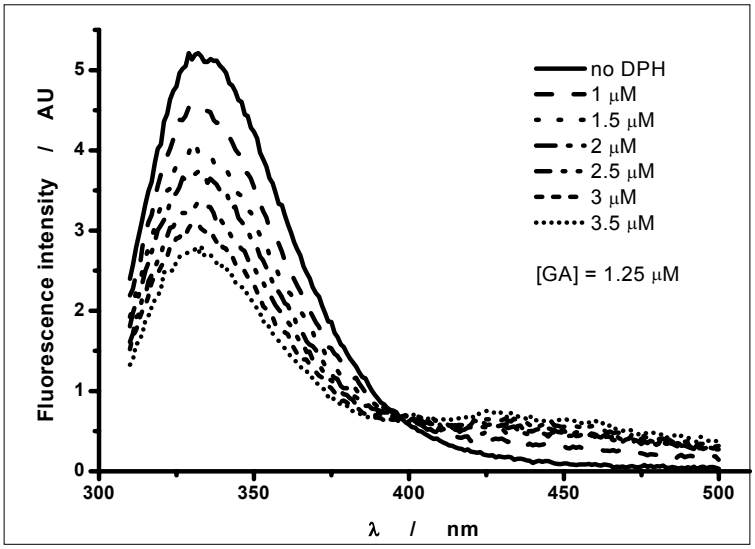

Fig. 2. FRET spectra for various acceptor concentrations recorded on liposomes doped with GA and TMA-DPH.

Figure 2 presents the spectra of liposomes doped with GA and different values of TMA-DPH concentration resulted after the correction procedure described in the Materials and Methods section. The decrease of Trp emission (the peak in the range of 320–350 nm) simultaneously with the increase of TMA-DPH emission (420–500 nm) for increasing values of acceptor concentration is a clear evidence of FRET occurring.

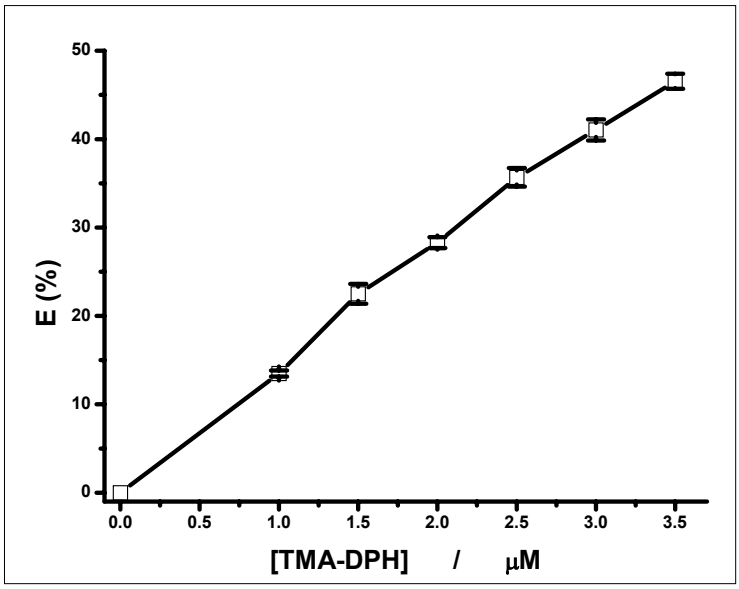

Fig. 3. FRET efficiency against acceptor concentration for liposomes doped with GA and TMA-DPH.

In the second part of our study we have applied the FRET procedure to test the insertion of OmpF into the lipid bilayer of SUV.

The absorption and emission spectra of Trp, OmpF and DPH are plotted in Figure 4. In spite of the blue shift of OmpF emission peak (maximum located at 313 nm) there is still an enough large overlapping to assure the main FRET condition to be fulfilled.

In Figure 5 the FRET spectra (corrected as described above) recorded on liposomes with OmpF and acceptor are presented. As in the case of GA the Trp emission decreases and the DPH signal increases simultaneously proving the energy transfer.

The computed efficiency for this type of experiment is plotted in Figure 6 against the TMA-DPH concentration.

A significant value of FRET occurs for the OmpF and TMA-DPH pair of molecules, close to that found in the GA experiments, allowing us to consider that the OmpF molecules have been inserted in the liposomes membrane.

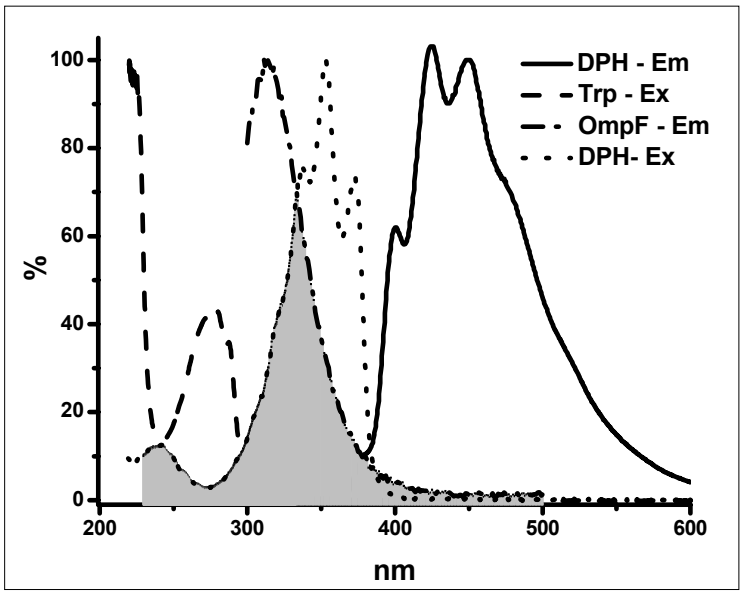

Fig. 4. Normalized absorption and emission spectra of Trp, OmpF and DPH.

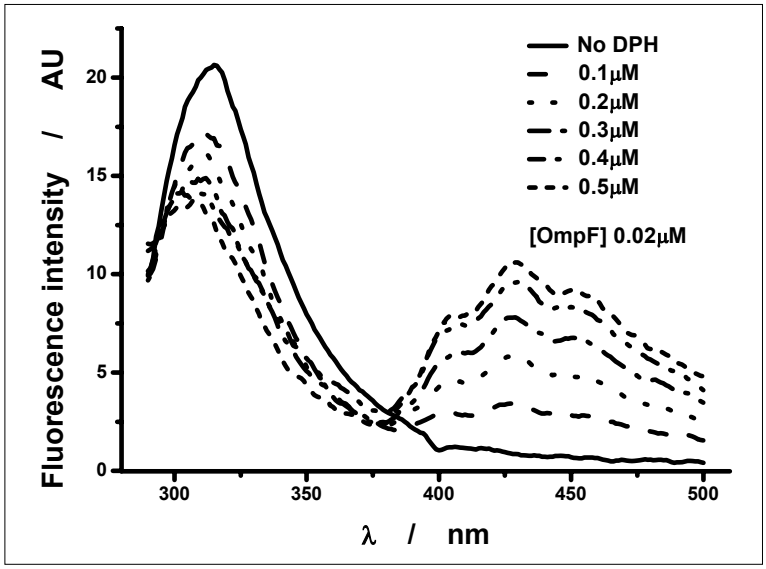

Fig. 5. FRET spectra for various acceptor concentrations recorded on liposomes doped with OmpF and TMA-DPH.

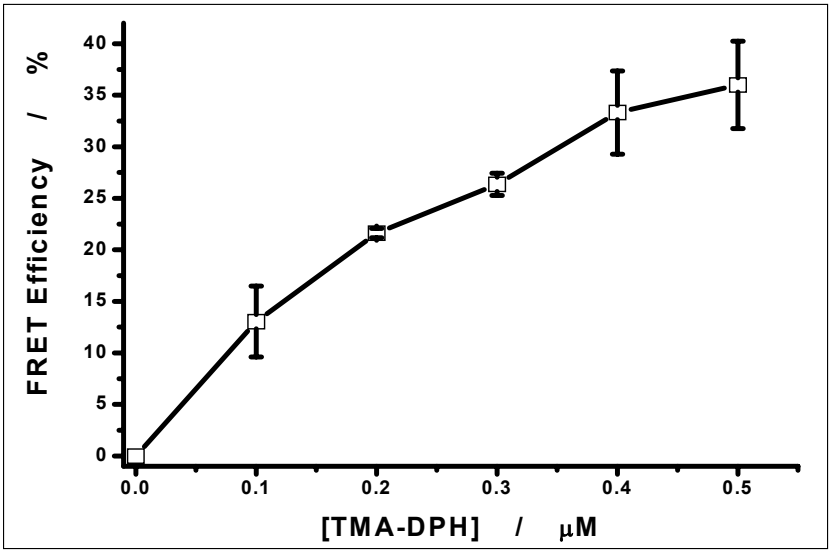

Fig. 6. FRET efficiency against acceptor concentration for liposomes doped with OmpF and TMA-DPH.

DISCUSSIONS

The results presented above suggest that the OmpF molecules are inserted in the lipid bilayer, but a more powerful analysis of data may provide quantitative evidences for this hypothesis. A large class of theoretical models was developed to compute the FRET efficiency for the interaction among donors and acceptors spread on a surface. The target was to describe in a reasonable way the phenomenon occurring in a membrane [1, 10]. We use the simplest model to analyze our data because the intention was only to find an approximate evaluation of the distance between donor and acceptor at which FRET still occurs. A measure of this distance is the Forster radius (the distance at which the efficiency is 50% for a certain donor acceptor pair). Supposing the donors and acceptors uniformly spread on a plane surface and integrating the contributions of all possible pairs of donor acceptor, a simple relation may be inferred for the FRET efficiency [10]. In this simple model the smallest distance between the donor and acceptor was approximated to zero. The analytic solution of this problem is:

$$E = 1 - \sum_{j=0}^{\infty} [(-\varepsilon C)^j \Gamma(j/3+1)/j!] \tag{2}$$

where $\varepsilon = \pi\Gamma(2/3)$ and $C = R_0^2 c$, R_0 being Forster radius and c the acceptor surface concentration.

Simulating the efficiency for our experimental conditions (the surface acceptor concentration was estimated considering ~ 0.6 nm^2 the average surface for one lipid molecule in the lipid bilayer of SUV) and for some R_0 values we obtained the plots from Figures 7 and 8.

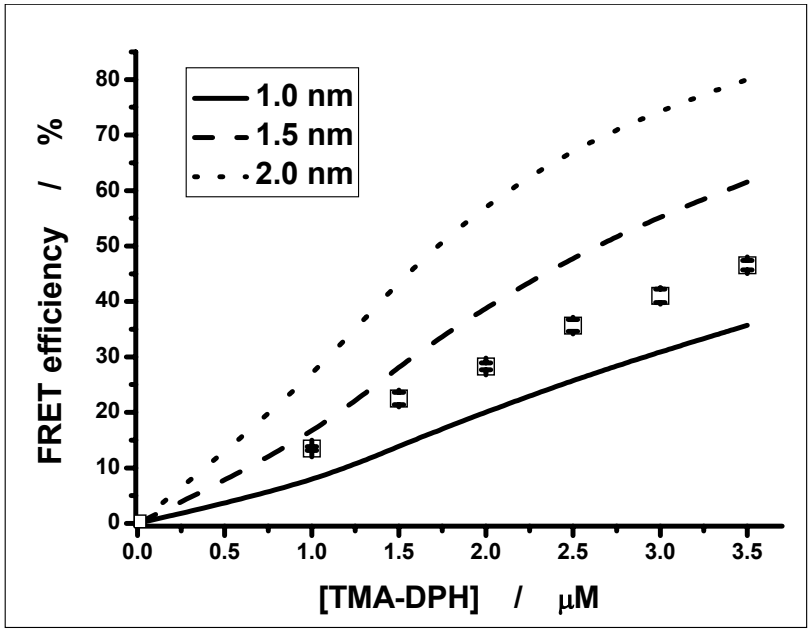

Fig. 7. Simulated FRET efficiency in comparison with experimental data for the GA experiments.

Figure 7 shows the FRET efficiency for GA experiments. Comparing these data with the efficiency computed from experimental data, a reasonable fit is obtained for 1.2 nm. The GA molecules inserted in the bilayer have at least a part of the Trp residues well exposed to the hydrophobic core of the membrane [6].In this case, the average value of the smallest distance between Trp from GA and TMA-DPH can be estimated as being pretty similar with half of the average distance between two adjacent lipid molecules (~ 1.3 nm), the GA molecule having a slower lateral diffusion compared with lipids diffusion. The R_0 value resulted from the simulation fits reasonably to, this value confirming the hypothesis.

A similar simulation was done for OmpF experiments and the results are plotted in Figure 8.

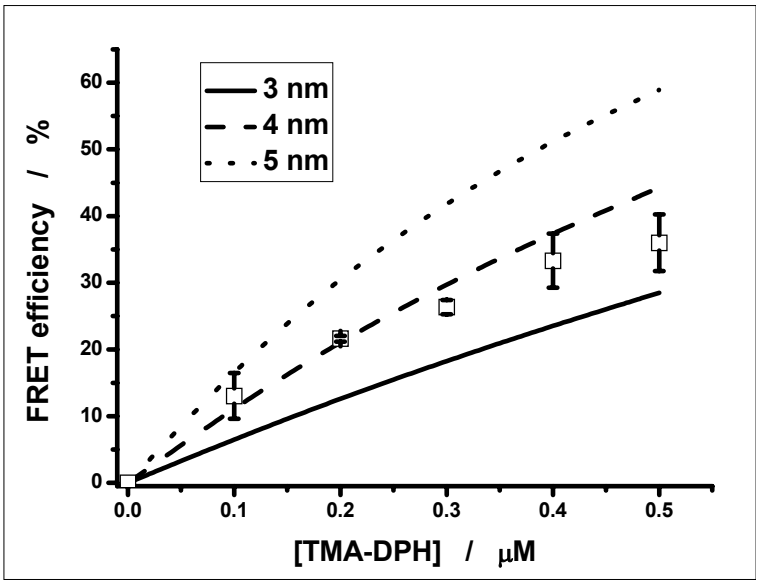

Fig. 8. Simulated FRET efficiency in comparison with experimental data for the OmpF experiments.

Comparison with the experimental data resulted in an estimated value of 3–4 nm for R_0. There are two Trp residues in an OmpF monomer. One of them is located at the lipid protein interface and the other one at the contact between two adjacent monomers [5]. Considering the pore radius of 1 nm [7] we can estimate a distance of ~ 3 nm between the second Trp residue and the closest acceptor molecule. The first Trp residue being very close to the acceptor area, a higher average R_0 value results compared to the GA case. From this evaluation we may conclude that the OmpF molecules are inserted in the membrane.

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