

# Membrane Protein–Lipid Selectivity: Enhancing Sensitivity for Modeling FRET Data

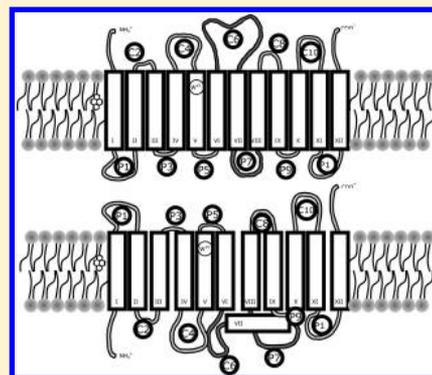
Carme Suárez-Germà,<sup>‡,†</sup> Luís M. S. Loura,<sup>||</sup> Manuel Prieto,<sup>⊥</sup> Òscar Domènech,<sup>‡,†</sup> M. Teresa Montero,<sup>‡,†</sup> Arturo Rodríguez-Banqueri,<sup>§</sup> José L. Vázquez-Ibar,<sup>§</sup> and Jordi Hernández-Borrell<sup>‡,†,\*</sup>

<sup>†</sup>Departament de Fisicoquímica, Facultat de Farmàcia, UB and <sup>‡</sup>Institut de Nanociència i Nanotecnologia IN<sup>2</sup>UB, <sup>§</sup>ICREA and Institut de Recerca Biomèdica, Parc Científic de Barcelona, 08028 Barcelona, Spain

<sup>⊥</sup>Centro de Química-Física Molecular and IN, IST, 1049-001, Lisboa, Portugal

<sup>||</sup>Faculdade de Farmácia, Universidade de Coimbra, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal and Centro de Química de Coimbra, 3004-535 Coimbra, Portugal

**ABSTRACT:** Förster resonance energy transfer (FRET) is a powerful method for the characterization of membrane proteins lipid selectivity. FRET can be used to quantify distances between a single donor and a single acceptor molecule; however, for FRET donors and acceptors scattered in the bilayer plane, multiple donor–acceptor pairs and distances are present. In addition, when studying protein/lipid selectivity, for a single tryptophan used as a donor; several lipid acceptors may be located at the boundary region (annular lipids) of the protein. Therefore, in these experiments, a theoretical analysis based on binomial distribution of multiple acceptors around the membrane proteins is required. In this work, we performed FRET measurements between single tryptophan lactose permease (W151/C154G LacY) of *Escherichia coli* and pyrene-labeled phospholipids (Pyr-PE, Pyr-PG, and Pyr-PC) reconstituted in palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-choline, and 1,2-dioleoyl-*sn*-glycero-3-phospho-choline at 25 and 37 °C. To increase the sensitivity of the method and to ascertain the lipid selectivity for LacY, we reconstituted the protein in the pure phospholipids doped with 1.5% of labeled phospholipids. From fitting the theoretical model to the experimental FRET efficiencies, two parameters were calculated: the probability of a site in the annular ring being occupied by a labeled pyrene phospholipid and the relative association constant between the labeled and unlabeled phospholipids. The experimental FRET efficiencies have been interpreted taking into account the particular folding of the protein in each phospholipid matrix. Additional information on the annular lipid composition for each system has been obtained by exciting W151/C154G LacY and monitoring the emission intensities for monomer and excimer of the pyrene spectra. The results obtained indicate a higher selectivity of LacY for PE over PG and PC and pointed to a definite role of the acyl chains in the overall phospholipid–protein interaction.



## INTRODUCTION

Cell envelopes play an important role in many physiological and pathological processes: signal transduction; transport of drugs and metabolites; energy generation; and development of tissues, including tumor metastasis and viral and bacterial infections, among many others. The cell membrane is presently viewed as a heterogeneous object because of the lateral distribution and segregation of its two fundamental components: phospholipids and proteins. Transmembrane proteins (TMPs) involved in specific transport molecules across the phospholipid bilayer represent 5–10% and 3% of total proteins encoded by bacterial and human genomes, respectively.

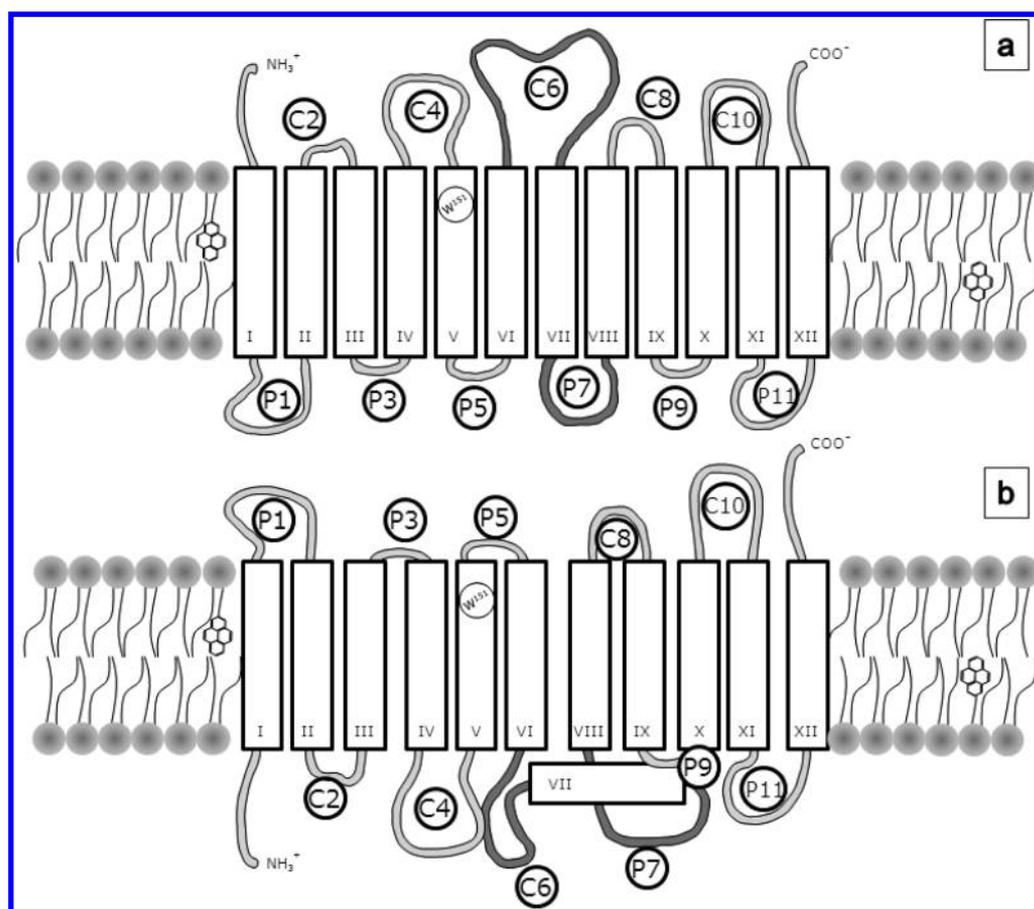
A large number of secondary transporters, in which the source of energy for the process of transport depends on the electrochemical potential gradient ( $\Delta\mu_i$ ), of ions such as  $\text{Na}^+$  or  $\text{H}^+$  contain 12 or 14 transmembrane segments (TMS) ( $\alpha$ -helix),<sup>1</sup> crossing the membrane in a zigzag fashion. Many of these proteins play an important role in conferring resistance to

drugs (anticancer and antibiotics) in both bacterial and eukaryotic cells. One of the paradigmatic models of membrane transporters is lactose permease (LacY) of *Escherichia coli*.<sup>2</sup> LacY, the secondary structure of which is shown in Figure 1a, is probably the best characterized of all proteins belonging to the 12-TMS group that also includes, among others, the efflux pumps LmrP of *Lactococcus lactis* and NorA of *Staphylococcus aureus*,<sup>3</sup> which actively expel daunomycin and norfloxacin, respectively. In the context of the chemiosmotic theory,<sup>4</sup> LacY utilizes the Gibbs energy stored in  $\Delta\mu_{\text{H}^+}$  to drive the uphill translocation of galactosides. Although the coupling mechanism is not completely solved, the basic pathway of sugar and  $\text{H}^+$  translocation through LacY and across the membrane is known in high detail.<sup>2</sup> TMPs are solvated by membrane lipids. There is

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**Figure 1.** Secondary structure model of lactose permease showing topological organization in the presence (a) and absence (b) of PE or PC. Putative transmembrane helices, connected by extramembrane loops, cytoplasmic (C) and periplasmic (P), are shown in boxes. The approximate positions of the W151 (donor) (helix V) and the position of pyrene labeled molecules (acceptor) in the annular region is also shown. Based on ref 19.

evidence, mainly based on earlier electron spin resonance experiments,<sup>5</sup> supporting the existence of a layer of phospholipids in intimate interaction with the TMPs that are known as annular lipids. In physical terms, this is a boundary region that provides an adequate thickness and lateral pressure to embed the protein following what is referred to in the field as the matching principle.<sup>6</sup>

In previous works,<sup>7,8</sup> we have demonstrated that an adequate method to investigate the composition of the annular region is Förster resonance energy transfer (FRET).<sup>9</sup> The strategy consists of measuring the efficiency of the energy transfer between a single tryptophan (Trp) mutant of LacY (W151/C154G),<sup>10</sup> used as a donor (D), and different pyrene-labeled phospholipids as acceptors (A). The main conclusions drawn were that both phosphoethanolamine (PE) and phosphoglycerol (PG) can be part of the annular region, with PE being the predominant phospholipid. On one hand, these results reinforce the basic consensus on the PE requirement for LacY correct folding and *in vivo* function.<sup>11</sup> On the other hand, to better mimic the inner bacterial membrane composition, these previous efforts were carried out mostly in PE/PG 3:1 mixtures, a ratio identical to that found in the inner membrane of *E. coli*.<sup>12</sup>

This creates a dilution problem. For example, in an experiment in which the acceptor is labeled PE, even if the annular region would consist solely of PE lipid, the enrichment of labeled PE in this layer would be only of a factor 4/3. Adding

to the fact that unspecific FRET to acceptors outside the annular layer is always present, this would imply a rather modest increase in the expected FRET efficiency. Finally, the simple fact that a pyrene acyl chain labeled lipid behaves identically to an unlabeled lipid of the same class is questionable, and this cannot be resolved in an experiment in which the host lipid matrix is a two-component mixture. For these reasons (to gain increased sensitivity and to assess the extent of correct reporting by the acceptor probes of each class), this protein system is readdressed in this paper, using different one-component host lipid matrixes. In addition, the influence of the headgroup and acyl chain composition is investigated.

In this work, we have reconstituted single-W151/C154G LacY in proteoliposomes formed with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-choline (POPC) or 1,2-dioleoyl-*sn*-glycero-3-phospho-choline (DOPC). The FRET strategy in the framework of this study consisted in measuring the efficiency of the energy transfer (*E*) between an engineered single tryptophan situated in a hydrophilic cavity in the center of LacY and three different pyrene-labeled phospholipids used as acceptors that are analogues of PE, PG, and PCs.

## EXPERIMENTAL METHODS

**Materials.** *N*-Dodecyl- $\beta$ -D-maltoside (DDM) was purchased from Anatrace (Maumee, OH, USA). POPE, POPG, POPC, and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (Pyr-PC), 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol ammonium salt (Pyr-PG), 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine ammonium salt (Pyr-PE), and fluorescein-5-maleimide were purchased from Invitrogen (Barcelona, Spain).  $\beta$ -D-Galactopyranosyl-1-thio- $\beta$ -D-galactopyranoside (TDG), isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and Bio-Beads SM-2 were purchased from Bio-Rad (Hercules, CA, USA). All other common chemicals were ACS grade.

**Bacterial Strains and Protein Purification.** These detailed procedures have been described in previous papers.<sup>7,8</sup> Briefly, *E. coli* BL21(DE3) cells (Novagen, Madison, WI, USA) transformed with plasmid pCS19 encoding single-W151/C154G LacY provided by Dr. H. Ronald Kaback (UCLA, USA), were grown in Luria–Bertani broth at 30 °C containing ampicillin (100  $\mu$ g/mL) and induced at the appropriate moment with 0.5 mM IPTG. Cells were disrupted, and the membrane fraction was harvested by ultracentrifugation. Membranes were solubilized by adding DDM and purified by Co(II) affinity chromatography (Talon Superflow, Palo Alto, CA, USA). Protein eluted with 150 mM imidazole was subjected to gel filtration chromatography using a Superdex 200 20/30 column (GE-Healthcare, UK) equilibrated with 20 mM Tris–HCl (pH 7.5), 0.008% DDM. The protein was concentrated by using Vivaspin 20 concentrators (30 kDa cutoff; Vivascience, Germany) and stored on ice. Protein identification was performed by SDS/PAGE electrophoresis, and protein quantitation was carried out using a micro-BCA kit (Pierce, Rockford, IL).

**Vesicle Preparation and Protein Reconstitution.** Liposomes and proteoliposomes were prepared according to methods published elsewhere.<sup>7,8</sup> Briefly, chloroform–methanol (2:1, vol/vol) solutions containing appropriate amounts of both labeled and unlabeled phospholipids were dried under a stream of oxygen-free N<sub>2</sub> in a conical tube. The total concentration of phospholipids was calculated as a function of the desired lipid-to-protein ratio and protein concentration (1.5  $\mu$ M). The amount of fluorescent probe was  $x = 0.015$  for all the experiments. The resulting thin film was kept under high vacuum for  $\sim 3$  h to remove any traces of organic solvent. Multilamellar liposomes were obtained following redispersion of the film in 20 mM Hepes, 150 mM NaCl buffer, pH 7.40, and applying successive cycles of freezing and thawing both below and above the phase transition of the phospholipids and sonication for 2 min in a bath sonicator. Afterward, large, unilamellar liposomes supplemented with 0.2% of DDM were incubated overnight at room temperature. Liposomes were subsequently mixed with the solubilized protein and incubated at 4 °C for 30 min with gentle agitation to obtain a lipid-to-protein ratio (w/w) of 40. DDM was extracted by addition of polystyrene beads (Bio-Beads SM-2, Bio-Rad).

**Binding Properties of Single-W151/C154G LacY Reconstituted in Vesicles.** Substrate recognition by single-W151/C154G LacY reconstituted in lipid vesicles was tested by adapting a previously described protocol<sup>7,8</sup> based on the

protection of the substrate against thiol modification of LacY. Briefly, 50  $\mu$ L of proteoliposomes containing 1.5  $\mu$ M of single-W151/C154G LacY<sup>13</sup> were incubated at room temperature for 5 min with either TDG or 15 mM L-glucose. Next, the samples were incubated with the fluorescent dye fluorescein-5-maleimide for 10 min at room temperature. The reaction was stopped by adding 5 mM of DTT. To evaluate the extent of LacY labeling, proteoliposomes were solubilized with 1% SDS and subjected to 12% PAGE gel electrophoresis. In-gel fluorescence was evaluated using a G-BOX gel analysis instrument (Syngene, Cambridge, UK) and compared with the total amount of protein after staining the same gel with Coomassie blue.

**FRET Methodology.** Steady state fluorescence measurements were carried out with an SLM-Aminco 8100 (Urbana, IL, USA) spectrofluorometer. The cuvette holder was thermostatted with a circulating bath (Haake, Germany), which was used to control temperature within 0.1 °C. The fluorescence experiments were performed at 25 and 37 °C. The excitation and emission bandwidths were 4/4 and 8/8 nm, respectively. Annular fluidity was determined as described elsewhere.<sup>14</sup> Pyrene was excited at 338 nm, with fluorescence spectra scanned from 350 to 500 nm. For energy transfer measurements, Trp was excited at 295 nm, and the spectra were recorded from 300 to 500 nm. To calculate the excimer-to-monomer fluorescence ratio (E/M), we used signal intensities at 375 nm (corresponding to the peak of monomer band) and 470 nm (maximum of pyrene excimer band). As described in detail elsewhere,<sup>7,8</sup> single-W151/C154G LacY, the donor, was excited at 295 nm, and emission of the pyrene-labeled phospholipids, the acceptor, was monitored at 338 nm.

FRET efficiencies ( $E$ ) are calculated according to the equation

$$E = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\int_0^{\infty} i_{DA}(t) dt}{\int_0^{\infty} i_D(t) dt} \quad (1)$$

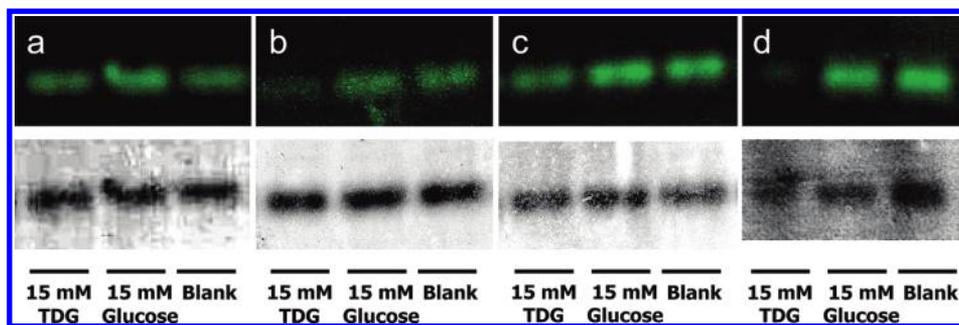
where  $I_D$  and  $I_{DA}$  are the tryptophan emission intensities in the absence or presence of pyrene acceptors, respectively. The reported values of experimental  $E$  are the averages of triplicate measurements from five separate reconstitutions. In the case of transmembrane proteins, we have to consider the existence of two different populations of phospholipids, those forming the first shell surrounding the protein, confined in the so-called boundary region, and those of the bulk. Assuming these two populations of A molecules, the fluorescence decay of D molecules can be written as

$$i_{DA}(t) = i_D(t) \rho_a(t) \rho_r(t) \quad (2)$$

where  $i_D$  and  $i_{DA}$  are the donor fluorescence decays in absence and presence of acceptor molecules, respectively. Since the number of annular pyrene phospholipids around each protein molecule is expected to follow a binomial population,<sup>15</sup> the annular contribution to the decay can be expressed as

$$\rho_a(t) = \sum_{n=0}^m e^{-nk_t t} \binom{m}{n} \mu^n (1 - \mu)^{m-n} \quad (3)$$

where  $m$  is the number of phospholipid molecules in the first layer surrounding the protein, taken as 46 for LacY;<sup>8</sup>  $\mu$  is defined as the probability of each site in the annular ring being



**Figure 2.** Substrate recognition by single-W151/C154G LacY reconstituted in proteoliposomes. Fluorescein–maleimide labeling of purified single-W151/C154G LacY reconstituted in vesicles composed of (a) POPE, (b) POPG, (c) POPC, and (d) DOPC. As indicated, the experiments were performed in the presence of 15 mM of TDG, 15 mM L-glucose, or no substrate (control). The upper panels (black background) correspond to the fluorescence intensity of fluorescein-labeled protein after being subjected to a 12% SDS–PAGE gel electrophoresis. The lower panels are the same gels after protein staining with Coomassie blue.

occupied by a labeled pyrene phospholipid; and  $k_t$  is the rate constant for D–A energy transfer,

$$k_t = \frac{1}{\tau} \left( \frac{R_0}{R} \right)^6 \quad (4)$$

where, in turn,  $\tau$  is the donor lifetime and  $R_0$  is the Forster radius (3.0 nm for the Trp/pyrene).<sup>16</sup> On the other hand,  $R$ , the distance between the D and annular A molecules, can be estimated according to

$$R = (w^2 + R_e^2)^{1/2} \quad (5)$$

where  $w$  (estimated as 1.2 nm) is the transverse distance between D (the Trp residue, for which an interfacial location is expected) and A (hydrophobic fluorophore, expected to reside near the bilayer center), and  $R_e$  (estimated as 3.0 nm) is the exclusion distance along the bilayer plane between the protein axis and the annular lipid molecules. For this system, the resulting value  $R = 3.2$  nm was considered.<sup>8</sup>

The probability,  $\mu$ , can be written as

$$\mu = K_s \frac{n_{\text{pyr}}}{n_{\text{pyr}} + n_{\text{PL}}} = K_s X_{\text{pyr}} \quad (6)$$

where the  $n$ 's are the mole numbers of the labeled ( $n_{\text{pyr}}$ ) and nonlabeled ( $n_{\text{PL}}$ ) phospholipids,  $X_{\text{pyr}}$  is the label mole fraction, and  $K_s$  is the relative association constant between the labeled and unlabeled phospholipids. Thus,  $K_s = 1$  denotes equal probability of finding acceptors in the annular region and in the bulk, whereas  $K_s = 0$  means no acceptor in the annular region.

Alternatively

$$\mu = \frac{n_{\text{pyr}}^{\text{ann}}}{n_{\text{pyr}}^{\text{ann}} + n_{\text{PL}}^{\text{ann}}} = X_{\text{pyr}}^{\text{ann}} \quad (7)$$

By inserting eq 7 into eq 6, we obtain a more intuitive meaning of  $K_s$ ,

$$X_{\text{pyr}}^{\text{ann}} = K_s X_{\text{pyr}} \quad (8)$$

that is,  $K_s$  is the ratio between the acceptor mole fractions in the annular region and in the overall system.

The FRET contribution of acceptors randomly distributed outside the annular region is given by Davenport et al.<sup>17</sup> as

$$\rho_r(t) = \exp \left\{ -4n_2\pi l^2 \int_0^{1/\sqrt{l^2 + R_e^2}} \frac{1 - \exp(-tb^3\alpha^6)}{\alpha^3} d\alpha \right\} \quad (9)$$

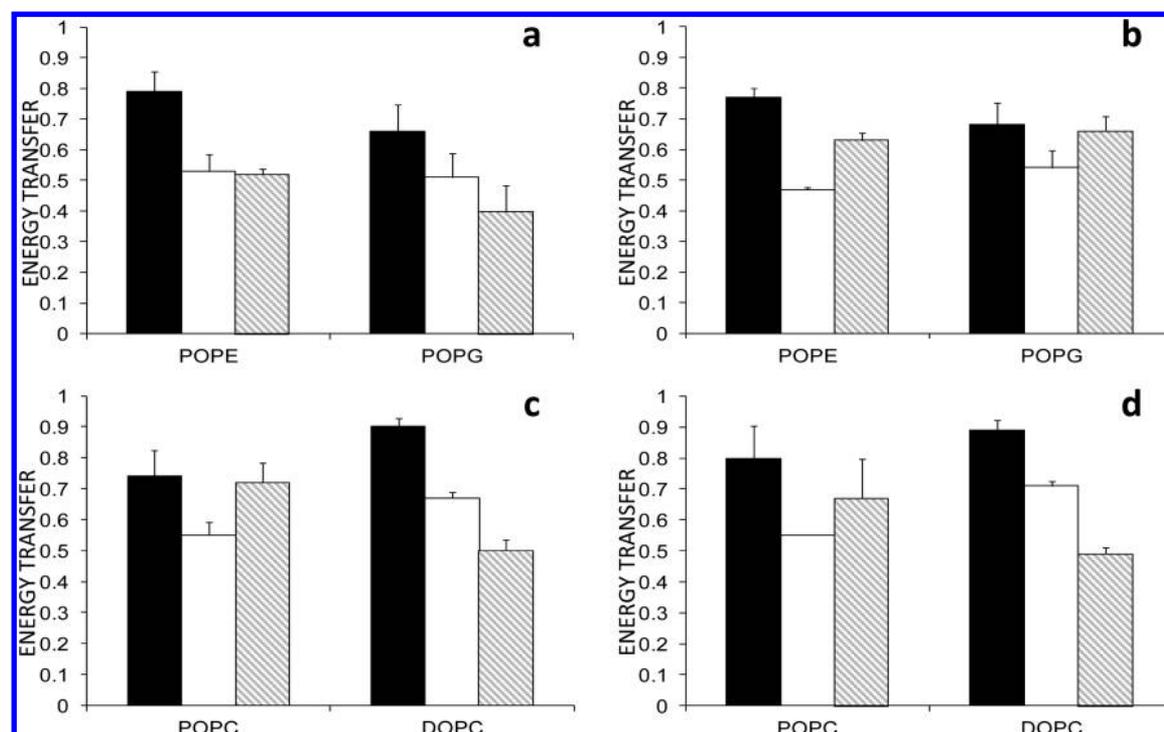
where  $b = (R_0/l)^2\tau^{-1/3}$ ,  $n_2$  is the acceptor density in each leaflet, and  $l$  is the distance between the plane of the donors and the plane of the acceptors.

## RESULTS AND DISCUSSION

The topological organization of LacY is well established.<sup>18</sup> As can be seen in Figure 1, the protein in its natural topology consists of 12 transmembrane  $\alpha$ -helices, crossing the membrane in a zigzag fashion, that are connected by 11 relatively hydrophilic, periplasmic (P) and cytoplasmic (C) loops, with both amino and carboxyl termini on the cytoplasmic surface. It is important to note within the context of the following discussion that in cells and proteoliposomes formed with PG or cardiolipin, LacY adopts an inverted topology (Figure 1b), whereas in the ones containing PE or PC, LacY adopts the natural topology (Figure 1a).<sup>19</sup> In this work, for FRET modeling, LacY has been assimilated to a cylinder with a diameter  $\sim 6$  nm in which the single tryptophan residue (W151) lies in the center of the cylinder. Despite the limitations of such approach, the model was tested successfully in preceding works,<sup>8</sup> where it was assumed that the single-W151/C154G mutant of LacY is indistinguishable from the global structure of the wild type protein.

With this assumption, we have reconstituted the protein in matrixes including 1.5% of pyrene-labeled phospholipids. This increase in the unlabeled/labeled phospholipid ratio was intended to improve the sensitivity of the FRET measurements to produce efficiencies of  $E \approx 0.5$ , for which FRET sensitivity to distance is maximal. Since LacY topological organization is sensitive to the lipid environment,<sup>19</sup> it is quite relevant to study the structural organization of the protein when reconstituted in the one-component host lipid matrixes used in the present work.

To ascertain the folding of LacY in the different matrixes, we used a fluorescence experiment that provides information about the ability of LacY to recognize its specific substrate, TDG.<sup>8</sup> Binding of TDG to LacY protects against the covalent modification of the protein by the fluorescent dye fluorescein–maleimide, whereas the nonsubstrate, L-glucose, does



**Figure 3.** FRET efficiency between W151 and Pyr-PE (black columns), Pyr-PG (white columns) and Pyr-PC (striped columns) in POPE and POPG matrixes at 25 (a) and 37 °C (b) and in POPC and DOPC matrixes at 25 (c) and 37 °C (d). Proteoliposomes (1.5  $\mu$ M LacY) were doped with 1.5% of the corresponding phospholipids analog label. The error bars stand for  $\sigma/\sqrt{n}$ ,  $\sigma$  being the standard deviation, and  $n$ , the number of measurements performed.

**Table 1.** Experimental Efficiencies, Probabilities of Each Site in the Annular Ring Being Occupied by a Pyrene Labeled Phospholipid and Relative Association Constant toward LacY

	labeled lipid (1.5%)	POPE matrix				POPG matrix			
		experimental $E$	$\mu$	$K_s$	$K_s/K_s(\text{PE})$	experimental $E$	$\mu$	$K_s$	$K_s/K_s(\text{PE})$
25 °C	Pyr-PE	0.79	0.10	6.53	1.00	0.66	0.03	2.00	1.00
	Pyr-PG	0.53	0.00	0.00	0.00	0.51	0.00	0.00	0.00
	Pyr-PC	0.52	0.00	0.00	0.00	0.40	0.00	0.00	0.00
37 °C	Pyr-PE	0.77	0.08	5.53	1.00	0.68	0.04	2.47	1.00
	Pyr-PG	0.47	0.00	0.00	0.15	0.54	0.00	0.00	0.00
	Pyr-PC	0.63	0.02	1.40	0.25	0.66	0.03	2.00	0.81

not show such protection. As can be seen in Figure 2, in addition to the exception of POPE 98.5% lipid composition, TDG binding to LacY reconstituted in all the studied lipid systems partially blocks fluorescein–maleimide labeling. This indicates that the reconstituted protein can selectively recognize the substrate TDG over the nonsubstrate L-glucose.<sup>2</sup> Incidentally, it may be noticed that POPG 98.5% composition shows, in general, paler fluorescence, which may be attributed to the repulsion encountered by the negatively charged fluorescein when approaching the negative matrix, which may somewhat hinder the probe–protein interaction.

Given the functional data shown previously, the absence of TDG protection against fluorescein–maleimide labeling in LacY reconstituted with POPE (Figure 2, upper panel a) is somehow contradictory with the fact that PE is the most abundant phospholipid species (70%) in the inner *E. coli* membrane.<sup>12</sup> It is noteworthy that LacY reconstituted into phospholipids extracted from *E. coli* cytoplasmic membranes composed mainly of PE (up to 75%) is fully functional and shows high levels of active transport.<sup>19</sup> Similar to what it has been reported in other works,<sup>20</sup> fluorescence spectra of LacY

reconstituted in PE proteoliposomes was indistinguishable from the proteoliposomes of other compositions. This suggests that the position of the fluorophore, sited near the binding site, remains unaltered and that the global structure is maintained. Then we may assume that LacY adopts a correct topology when reconstituted in 98.5% POPE proteoliposomes.

Regarding matrixes formed with anionic phospholipids, it is well established that LacY cannot carry out uphill but downhill substrate transport.<sup>21</sup> This is due to a change in the topological organization of domains C6 and P7 of LacY (Figure 1b).<sup>19</sup> Nevertheless, the binding site remains unaltered in POPG, since it supports downhill transports and, in our studies, substrate recognition (Figure 2, upper panel b). Furthermore, similar functional behavior (only downhill transport) is found when LacY is reconstituted in proteoliposomes mostly composed of the neutral zwitterionic phospholipid DOPC.<sup>19</sup> However, it has been demonstrated that the protein conserves its physiological topology (Figure 1a).

Figure 3 shows the experimental FRET efficiency for the three Pyr-labeled phospholipids used as acceptors (Pyr-PE, Pyr-PG, and Pyr-PC) in all host lipids at 25 and 37 °C. At first

**Table 2.** Experimental Efficiencies, Probabilities of Each Site in the Annular Ring Being Occupied by a Pyrene Labeled Phospholipid and Relative Association Constant toward LacY

	labeled lipid (1.5%)	POPC matrix				DOPC matrix			
		experimental $E$	$\mu$	$K_s$	$K_s/K_s(\text{PE})$	experimental $E$	$\mu$	$K_s$	$K_s/K_s(\text{PE})$
25 °C	Pyr-PE	0.74	0.07	4.31	1.00	0.90	0.28	18.50	1.00
	Pyr-PG	0.55	0.00	0.02	0.00	0.67	0.03	2.24	0.12
	Pyr-PC	0.72	0.05	3.63	0.84	0.50	0.00	0.00	0.00
37 °C	Pyr-PE	0.80	0.11	7.08	1.00	0.89	0.25	16.47	1.00
	Pyr-PG	0.55	0.00	0.02	0.00	0.71	0.05	3.60	0.22
	Pyr-PC	0.67	0.03	2.24	0.32	0.49	0.00	0.00	0.00

sight, there is a larger preference for Pyr-PE, irrespective of the temperature and the phospholipid matrix. Pyr-PE is preferred over Pyr-PG, and the latter over Pyr-PC, in the POPE and POPG matrixes at 25 °C (Figure 3a), and in the DOPC matrix at both temperatures (Figures 3a, c). A change in preference, Pyr-PC by Pyr-PG occurs in the POPE and POPG matrixes at 37 °C (Figure 3b) and in the POPC matrix at both temperatures (Figure 3c, d). Interestingly Pyr-PC shows a higher efficiency in POPC than in DOPC, indicating a possible influence of the degree of unsaturation of the acyl chains. An opposite behavior is observed for Pyr-PG, for which the efficiency in DOPC is higher than in the POPC matrix.

In Table 1, the experimental FRET values are listed along with the calculated  $\mu$  and  $K_s$  values. As can be seen, the probability of finding labeled phospholipids at the annular regions ( $\mu$ ) is always the highest, irrespective of the matrix, for Pyr-PE. This behavior reflects the values of the FRET efficiency mentioned above. By inspecting the outcome for  $K_s$  values, we notice that the largest values are obtained for Pyr-PE in all matrixes and both temperatures. It is worth mentioning that ideally,  $K_s = 1$  for any probe that mimics the nonlabeled phospholipids, and values between 1 and 3 have been reported.<sup>9</sup> Therefore, these high values of  $K_s$  obtained for Pyr-PE may indicate either an annular region extremely enriched in the label or that Pyr-PE does not mimic well the unlabeled phospholipid. Although this may be a handicap, if one compares across probes in the same host lipid, it becomes clear that there is an effect of preference of Pyr-PE for PE over PG and PC. Since the probes are all equal except for the headgroup, and for comparing the different probes in the same host lipid,  $K_s/K_s(\text{PE})$  ratios are provided in Table 1.

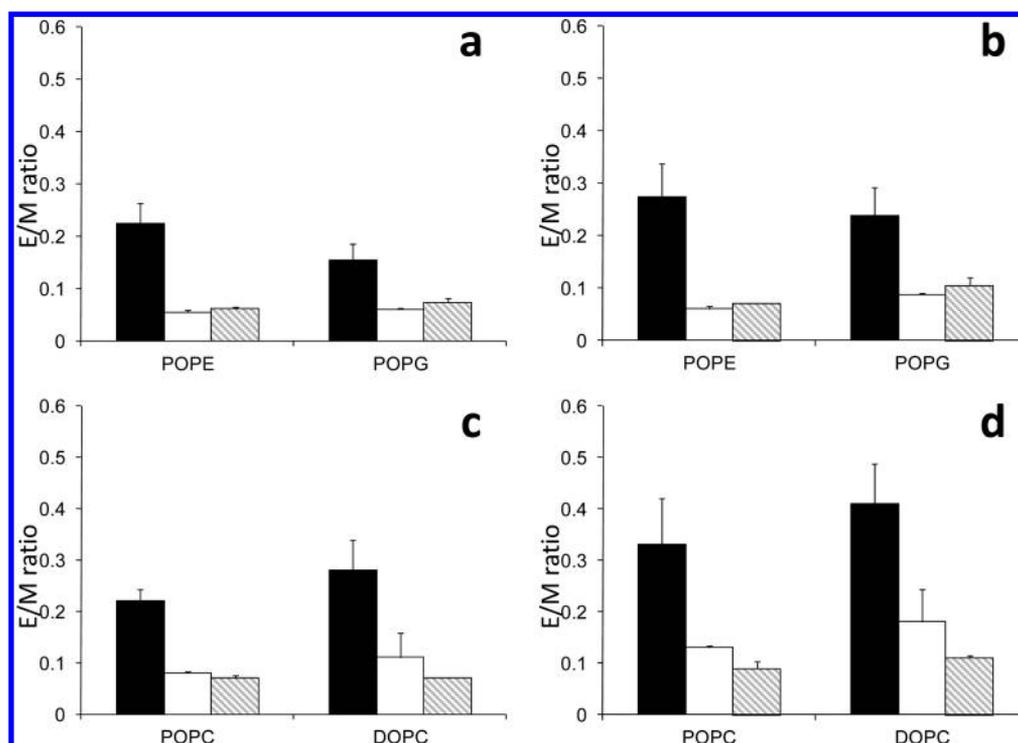
In the POPE matrix,  $\mu$  values indicate that Pyr-PG is excluded at both temperatures and that Pyr-PC is excluded at 25 °C and shows a small enrichment at 37 °C. Since  $K_s = 0$  means no acceptor in the annular region, it becomes clear that at 25 °C, Pyr-PG and Pyr-PC are completely excluded. Although Pyr-PG behaves in the same way at 37 °C, LacY shows an increased preference for Pyr-PC at this temperature. The overall results in the POPE matrix, in which LacY is folded closely to the in vivo conditions (Figure 1a), point to the fact that Pyr-PE should be in closer proximity than the other labels. On the other hand, notice that  $\mu$  and  $K_s$  for Pyr-PE in the POPG matrix are compatible with a moderate enrichment of the label in the annular region. Notice that Pyr-PG is depleted from the annular region at both temperatures when the host phospholipid is POPG. Similarly, we can observe that Pyr-PC is also depleted when hosted by POPG at 25 °C and that a very slight enrichment is observed at 37 °C.

All these observations may be likely related to the inverted topology of domains C6 and P7 of LacY (see Figure 1b) when reconstituted in POPG proteoliposomes.<sup>19</sup> Our FRET

measurements in POPE and POPG matrixes confirm the preference of LacY for PE and its probable predominance in the annular ring.<sup>7,8</sup> This may indirectly support a hypothetical interaction between the PE headgroup and some specific residue of the protein.<sup>23,24</sup> Importantly, recent observations have shown that uphill transport occurs in *E. coli*, in which PE has been completely exchanged by PC.<sup>25</sup> Since in PE and PC matrixes LacY exhibits its natural topology (Figure 1a), this intriguing observation points to a more complex molecular interaction between the protein and the annular phospholipids. Hence, FRET measurements in PC matrixes (Table 2) become of interest given the fact that despite its natural topology in these matrixes, LacY shows only downhill transport in DOPC proteoliposomes.<sup>19</sup> Pyr-PG is slightly enriched in the annular region when the matrix is DOPC ( $K_s > 1$ ) but is excluded from it in a POPC matrix ( $K_s \sim 0$ ). However, the most interesting result is possibly that, according to the  $K_s$  values, Pyr-PC is enriched in the annular region in a POPC matrix ( $K_s > 1$ ) and excluded from it in a DOPC matrix ( $K_s \sim 0$ ).

Given that DOPC and POPC share the same headgroup and have very similar hydrophobic lengths in the bilayer (2.48 nm for DOPC vs 2.54 nm for POPC),<sup>26</sup> this difference is probably related to the different specific curvature of the two lipid species. It has been reported that whereas proper topology of LacY depends on a dilution of high negative surface charge density (and hence, probably the decreased affinity of the protein for PG), rather than on spontaneous curvature ( $C_0$ ),<sup>27</sup> the latter appears to be crucial regarding uphill transport of lactose by LacY in vivo,<sup>28</sup> with negative curvature lipids such as PE being required.  $C_0$  (POPC) is essentially zero, and DOPC, due to its additional unsaturated acyl chain, has a negative specific curvature ( $C_0(\text{DOPC}) = -0.11 \text{ nm}^{-1}$ ).<sup>26</sup> Although its value is still far from the nonbilayer lipid DOPE ( $C_0(\text{DOPE}) = -0.35 \text{ nm}^{-1}$ ),<sup>26</sup> it may justify the preference of properly reconstituted LacY for DOPC rather than POPC, and hence, the differential behavior in DOPC and POPC matrixes regarding selectivity for labeled probes.

In addition, DOPC is also much closer to PE on hydration properties. The fact that an opposite trend is observed for Pyr-PG (Table 2) is suspicious and probably related to the above-mentioned improper organization of LacY in PG. Interestingly, according to theoretical calculations,<sup>29</sup> although repulsive contributions in the core of the membrane are similar ( $\sim 166$  bar and  $\sim 200$  bar for POPC and DOPC, respectively), the repulsive forces at the headgroups level are much higher for POPC ( $\sim 675$  bar) than for DOPC ( $\sim 266$  bar). Therefore, whatever the precise mechanism involved in the phospholipid–protein interplay may be, there would be a subtle balance among the forces evolved from physicochemical properties of the molecules.



**Figure 4.** E/M ratios from the annular labeled lipids: Pyr-PE (black columns), Pyr-PG (white columns) and Pyr-PC (stripped columns) of  $1.5 \mu\text{M}$  LacY proteoliposomes of POPE and POPG at  $25 \text{ }^\circ\text{C}$  (A) and  $37 \text{ }^\circ\text{C}$  (B); and POPC and DOPC at  $25 \text{ }^\circ\text{C}$  (C) and  $37 \text{ }^\circ\text{C}$  (D). The error bars stand for  $\sigma/\sqrt{n}$ ,  $\sigma$  being the standard deviation, and  $n$ , the number of measurements performed.

The excited-state pyrene molecules display two characteristic peaks in the fluorescence spectra, for the monomer and excimer, respectively. The specific E/M ratio will result in the collision rate of the pyrene molecules. Thus, additional information can be obtained by exciting the single tryptophan of W151/C154G LacY and monitoring the emission intensities for M and E of the pyrene spectra because only the labeled molecules surrounding the protein, annular lipids, will be selectively excited. Consequently, the E/M ratio will provide information on the annular lipid proportion for each system.

The E/M ratios reported by the pyrene probes in the corresponding host phospholipids under study are shown in Figure 4. As can be seen, these ratios were always higher for Pyr-PE than for the other labeled phospholipids, at both temperatures and all phospholipid matrixes. Notice that slight differences in the E/M ratios for Pyr-PG and Pyr-PC are observed in the POPE and POPG matrixes (Figure 4a, b) and that Pyr-PG shows slightly higher E/M ratios than Pyr-PC in the POPC and DOPC matrixes (Figure 4c, d). Although caution should be taken with such conclusion, on the basis of the assumption that labeled and unlabeled phospholipids behave similarly, these overall results are in agreement with the overall FRET observations and support the idea that LacY is preferentially surrounded by PE rather than by the other phospholipids and that acyl chains may play a definite role in the transport processes.

## CONCLUSIONS

In this work, we have observed by using single component systems that the selectivity of LacY for PE is much higher than that for either PC or PG. That is, with the limitations imposed by the model and the system itself, we do confirm that Pyr-PE is able to get closer to or, alternatively, spend more time next to

the LacY. We report also that when the phospholipids in the annulus of the LacY are zwitterionic (PE) or neutral (PC) heteroacids, the probability for anionic PG to be in close proximity is very low. The presented data also suggest that the nature of the hydrophobic moiety and the appropriate heterological combination of phospholipids may govern the optimal function of LacY.

## AUTHOR INFORMATION

### Corresponding Author

\*Phone: (+34) 934035986. Fax: (+34) 934035987. E-mail: jordihernandezborrell@ub.edu.

### Notes

The authors declare no competing financial interest.

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