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Lactose permease lipid selectivity using Förster resonance energy transfer

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ABSTRACT

The phospholipid composition that surrounds a membrane protein is critical to maintain its structural integrity and, consequently, its functional properties. To understand better this in the present work we have performed FRET measurements between the single tryptophan residue of a lactose permease *Escherichia coli* mutant (single-W151/C154G LacY) and pyrene-labeled phospholipids (Pyr-PE and Pyr-PG) at 37 °C. We have reconstituted this LacY mutant in proteoliposomes formed with heteroacid phospholipids, POPE and POPG, and homoacid phospholipids DOPE and DPPE, resembling the same PE/PG proportion found in the *E. coli* inner membrane (3:1, mol/mol). A theoretical model has been fitted to the experimental data. In the POPE/POPG system, quantitative model calculations show accordance with the experimental values that requires an annular region composed of approximately ~90 mol% PE. The experimental FRET efficiencies for the gel/fluid phase-separated DOPE/POPG system indicate a higher presence of PG in the annular region, from which it can be concluded that LacY shows clear preference for the fluid phase. Similar conclusions are obtained from analysis of excimer-to-monomer (E/M) pyrene ratios. To test the effects of this on cardiolipin (CL) on the annular region, myristoyl-CL and oleoyl-CL were incorporated in the biomimetic POPE/POPG matrix. The experimental FRET efficiency values, slightly larger for Pyr-PE than for Pyr-PG, suggest that CL displaces POPE and, more extensively, POPG from the annular region of LacY. Model fitting indicates that CL enrichment in the annular layer is, in fact, solely produced by replacing PG and that myristoyl-CL is not able to displace PE in the same way that oleoyl-CL does. One of the conclusions of this work is the fact that LacY inserts preferentially in fluid phases of membranes.

1. Introduction

Membrane proteins account for over 25% of total cell proteins. The cytoplasmic membrane of *Escherichia coli*, for instance, is believed to contain more than 200 protein types, of which 60 or more might be involved in transporting ions and molecules across the membrane. A transport classification system has been proposed for membrane transport proteins (http://www.tcdb.org). There we can distinguish among (i) primary transporters if they use the energy released from the ATP hydrolysis to drive accumulation or efflux (i.e., the ABC transporters) and (ii) secondary transporters when they transduce the Gibbs energy stored in an electrochemical ion gradient into substrate concentration gradients. Membrane transport proteins are relevant not only in prevailing physiological conditions but also in diseases such as depression, diabetes, or antibiotic resistance mediated by efflux pumps. Hence, the increased interest to investigate the structure, function, and mechanisms of these systems.

Lactose permease (LacY) of *E. coli* is one of the most intensively studied membrane protein and is often taken as a paradigm for secondary transport proteins [1]. LacY belongs to the major facilitator superfamily (MFS) of transporters and consists of 12 transmembrane α-helices, crossing the membrane in a zigzag fashion that are connected by 11 either periplasmic or cytoplasmic loops, with both amino and carboxyl termini on the cytoplasmic side.

LacY translocates one substrate (specifically disaccharides containing a D-β-galactopyranosyl ring) with one H^+ in a symport mechanism (cotransport). Thus, under physiological conditions, H^+ moves down its electrochemical ion gradient ($\Delta\tilde{\mu}_i$)[2] and drives the uphill translocation of the substrate (against a concentration gradient). In absence of $\Delta\tilde{\mu}_i$, downhill movement of substrate along

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[2] In the bioenergetic field, for the electrochemical gradient of H^+ the "proton-motive force", defined as $\Delta p = \Delta\tilde{\mu}_{H^+} / F$ is often used.

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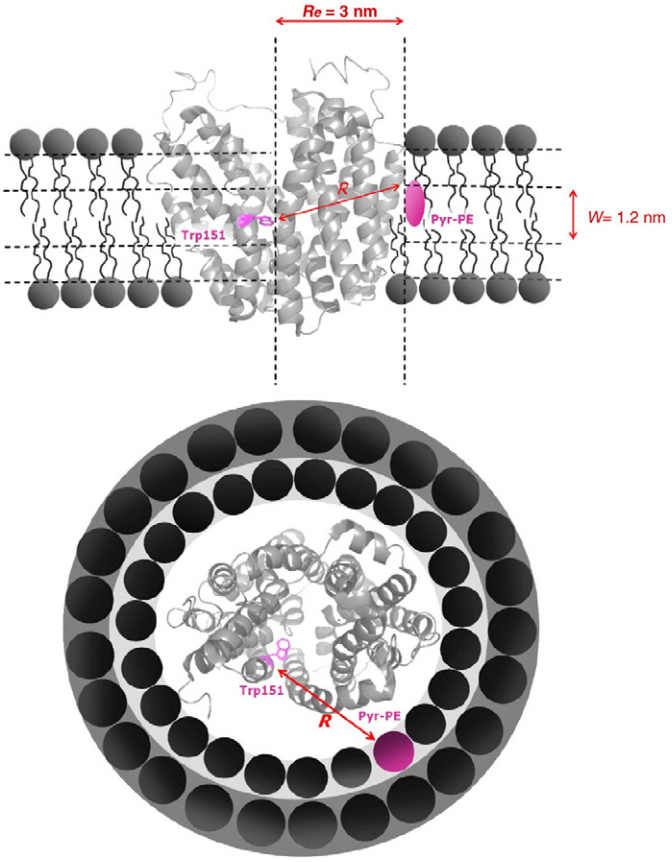

Fig. 1. Model of Lactose permease embedded in the bilayer: frontal view showing the transverse distance (w) between the W151 residue (donor) and the pyrene-labelled phospholipid acceptors (A) (Pyr-PE or Pyr-PG) (top) and sagittal view showing the two annular shell (bottom).

a concentration gradient drives uphill transport of H^+, leading to the generation of $\Delta\tilde{\mu}_i$, the polarity of which depends on the direction of the substrate concentration gradient. These phenomena are examples of vectorial coupling [3] with a 1:1 stoichiometry that takes place in a membrane protein. In the last years the three-dimensional structure of LacY obtained from X-ray diffraction (Fig. 1) [2] has revealed the architecture of the sugar binding site along with the position of the key residues implicated on sugar/H^+ coupling. A mechanism of energy transduction has been proposed based on the combination of the structural information and many biophysical and biochemical data [4].

The physiological activity of transmembrane proteins may be influenced by or dependent on the physicochemical properties of neighboring phospholipids [5,6]. Such dependence has been demonstrated in the case of, among others, the β-hydroxybutyrate dehydrogenase, the Ca^{+2}-ATPase or the melibiose permease from *E. coli*. In earlier works [7], it was pointed out that the amino group of phospholipids such as phosphatidylethanolamine (PE) is of crucial importance for LacY function. More recently, it has been conclusively demonstrated that LacY requires the presence of PE for its correct folding in the membrane during biogenesis [8], its function *in vivo* [9], and in maintaining its correct topology [10,11]. Likewise, the relevance of PE for other proteins as LmrP [12] or channels as KcsA [13] has been demonstrated, suggesting a general role of PE in membrane transport processes.

Although particular details are unknown, there are few doubts on how membrane lipids affect the structure and organization of membrane proteins [14]. As discussed elsewhere, the hydrophobic surface of membrane proteins is covered by a shell of phospholipids known as boundary or annular phospholipids [5]. Thus, the interplay between the phospholipids that constitute this region and membrane proteins is crucial for getting a complete view of the transport processes occurring in membranes. In physical terms, the boundary region should provide the adequate (i) length for the hydrophobic matching between the domain of the protein embedded in the bilayer and the phospholipids [6] and (ii) lateral pressure to seal the membrane during conformational changes experienced by the protein during transport events [15].

Electron spin resonance (ESR) [16] and Förster resonance energy transfer (FRET) [17,18] have been used for the investigation of the annular region. Based on ESR experiments, it is generally assumed that the phospholipids present are motionally restricted [19,20]. On the other hand, using FRET tools we have verified that the annular region of LacY in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine(POPE):1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) vesicles (3:1) is apparently enriched in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), but that 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) is also present [21]. Besides, the composition of the annular region varies in function of the particular POPE/POPG molar ratio, suggesting that the protein can take advantage of a given lipid composition to recruit most abundant lipid for its own needs.

In this work, we have reconstituted single-W151/C154G LacY in proteoliposomes formed with homoacid phospholipids 1,2-oleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-palmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) or heteroacid phospholipids, POPE and POPG, focusing on the same PE/PG proportion found in *E. coli* inner membrane [22]. The FRET strategy, in the framework of this study, consists of measuring the efficiency of FRET between the single tryptophan residue of single-W151/C154G LacY used as a donor, and two different pyrene-labeled phospholipids used as acceptors that are analogues of PG and PE, respectively. Using this experimental strategy, we have studied the influence that the different *sn*-2 acyl chains of PE, and cardiolipin (CL) presence on the annular region of LacY. The main objective of this article was to verify that the model derived by Fernandes et al. [17] previously used to rationalize FRET results from other membrane proteins fits to the results obtained with LacY, the representative of the 12-TMS.

2. Materials and methods

N-dodecyl-β-D-maltoside (DDM) was purchased from Anatrace (Maumee, OH, USA). 1,2-Palmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-oleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG), 1′,3′-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (myristoyl-CL), and 1′,3′-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (oleoyl-CL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol ammonium salt (Pyr-PG) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine ammonium salt (Pyr-PE) were purchased from Invitrogen (Barcelona, Spain). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was 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.

2.1. Bacterial strains and protein purification

Plasmid pCS19 encoding single-W151/C154G LacY with a 6-His tag at the C-terminus was generated as described [23] and provided by Dr. H. Ronald Kaback (UCLA, USA). *E. coli* BL21(DE3) cells (Novagen, Madison, WI, USA) transformed with this plasmid were grown in 6.4 L of Luria-Bertani broth at 30 °C containing ampicillin (100 μg/mL) to an absorbance (600 nm) of 0.6 and induced with

0.5 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were disrupted by passage through a French pressure cell, and the membrane fraction was harvested by ultracentrifugation. Membranes were solubilized by adding DDM to a final concentration of 2%, and LacY was 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. After column elution, the protein was concentrated by using Vivaspin 20 concentrators (30 kDa cutoff; Vivascience, Germany) and stored on ice. As determined by sodium dodecylsulfate/12% polyacrylamide gel electrophoresis followed by Coomassie blue staining, the preparations contained only a single band with an apparent molecular weight of 36 kDa. Protein was assayed by using a micro-BCA kit (Pierce, Rockford, IL).

2.2. Vesicle preparation and protein reconstitution

Liposomes and proteoliposomes were prepared according to methods elsewhere published [24]. Briefly, chloroform–methanol (3:1, vol./vol.) solutions containing appropriate amounts of both labelled and unlabeled phospholipids were dried under a stream of oxygen-free N_2 in a conical tube. The total concentration of phospholipids was calculated as a function of the desired lipid-to-protein ratio (LPR) and protein concentration (1.5 and 3 μM). The amount of fluorescent probe was $x = 0.0025$ for all the experiments. The resulting thin film was kept under high vacuum for approximately 3 h to remove organic solvent traces. Multilamellar liposomes (MLVs) were obtained following redispersion of the film in 20 mM HEPES, 150 mM NaCl buffer, pH 7.40, applying successive cycles of freezing and thawing below and above the phase transition of the phospholipids, and sonicating for 2 min in a bath sonicator. Afterwards, large unilamellar liposomes (LUVs) 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 an LPR (wt./wt.) of 40. DDM was extracted by addition of polystyrene beads (Bio-Beads SM-2; Bio-Rad) as described elsewhere [25].

2.3. Binding properties of single-W151/C154G LacY reconstituted in vesicles

Substrate recognition by single-W151/C154G LacY reconstituted in lipid vesicles was tested adapting a protocol previously described [23], based on the protection of the substrate against thiol modification of LacY. Briefly, 50 μL of proteoliposomes containing 1.5 μM of single-W151/C154G LacY were incubated at room temperature for 5 minutes with either, 15 mM β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG), 15 mM L-glucose or nothing. Next, the samples were incubated with the fluorescent dye Fluorescein-5-Maleimide (Invitrogen, USA) for 10 minutes at room temperature. The reaction was stopped by adding 5 mM of DTT. To evaluate the extent of LacY labelling, 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 to the total amount of protein after staining the same gel with Coomassie blue.

2.4. Fluorescence measurements

Steady state fluorescence measurements were carried out with an SLM-Aminco 8100 (Urbana, IL, USA) spectrofluorometer. The cuvette holder was thermostated with a circulating bath (Haake, Germany), which was used to control temperature within 0.1 °C. The fluorescence experiments were performed at 37 °C. The excitation and emission bandwidths were 4/4 and 8/8 nm, respectively. Bulk fluidity was determined as described elsewhere [26]. Pyrene fluorescence was excited at 338 nm, with fluorescence spectra scanned from 350 to 500 nm. For FRET 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). Based on the quenching of intrinsic tryptophan by pyrene phospholipids, the values of experimental FRET efficiency (E) were determined according to the equation

$$E = 1 - \frac{I_{DA}}{I_D} \tag{1}$$

where I_D and I_{DA} are the tryptophan emission intensities in the absence or presence of pyrene phospholipid derivative, respectively [27]. The I_D intensities were evaluated from the peak height of the 338 nm tryptophan fluorescence [28] under excitation at 295 nm, and the spectra were recorded from 300 to 400 nm. Quenching data were corrected for inner filter effects by following the procedure described elsewhere [29]. The reported values of E represent averages of triplicate measurements from five separate reconstitutions.

2.5. FRET modelling

The FRET results were rationalized using the formalism derived by Fernandes et al. [17] for a single trans-membrane α-helix and here adapted to a larger membrane-spanning protein. From geometrical considerations and molecular areas from compression isotherms of POPE [30], the number of phospholipid molecules in the first layer surrounding the protein of radius 3.0 nm [2] was estimated as 23 per bilayer leaflet or 46 in total. In these conditions, the donor fluorescence decay in presence of acceptor is given by:

$$i_{DA}(t) = i_D(t)\rho_{annular}(t)\rho_{random}(t) \tag{2}$$

where $i_D(t)$ is the donor decay in the absence of acceptor and $\rho_{annular}(t)$ and $\rho_{random}(t)$ are the FRET contributions due to acceptors located in the annular region and uniformly distributed beyond this region, respectively.

The former is given by [17]

$$\rho_{annular}(t) = \sum_{n=0}^{N} e^{-nk_T t}\binom{N}{n}\mu^n(1-\mu)^{N-n} \tag{3}$$

where N is the number of phospholipid molecules in the first layer surrounding the protein, μ is the probability of each of these phospholipids being labeled with acceptor fluorophore, and k_T is the rate coefficient for FRET between donors (located in this model at the axis of the cylindrical protein) and acceptors. All acceptor fluorophores are assumed to have the same transverse location, that is, the center of the bilayer. This is expected because pyrene is a hydrophobic label, and both molecular dynamics simulations [31] and experimental fluorescence data [32–34] have confirmed its location deep in the hydrocarbon region of the bilayer. In this way, the donor–acceptor distance can be taken as identical for all annular acceptors, irrespective of their membrane leaflet. Using $w = 1.2$ nm as the transverse distance between donors (Trp residue), for which an interfacial location is expected [35] and the bilayer center, and $R_e = 3.0$ nm as the exclusion distance along the bilayer plane between the protein axis and the annular lipid molecules, the donor–acceptor distance is given by $R = (w^2 + R_e^2)^{1/2} = 3.2$ nm, which, in turn, is used to calculate k_T by

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

to be inserted in Eq. (3) above. For the Förster radius (R_0) in Eq. (4), the value $R_0 = 3$ nm, reported for the Trp/pyrene pair [36], was used.

On the other hand, ρ_{random} is calculated using the relationship derived for FRET to acceptors located in a plane parallel to that of the donors [37]

$$\rho_{random}(t) = \exp\left\{-2n_2\pi w^2 \int_0^{w/R} \frac{1-\exp(-tb^3\alpha^6)}{\alpha^3} d\alpha\right\} \quad (5)$$

where $b = (R_0/w)^2\tau^{-1/3}$ and n_2 is the acceptor surface density (number of acceptors per unit area of the acceptor plane, calculated assuming that all pyrene fluorophores are located in the same plane, in the center of the bilayer). For calculation of n_2, molecular areas of 0.56 nm^2, 0.56 nm^2, and 1.26 nm^2 were assumed for POPE, POPG, and CL, respectively [38,39].

The theoretical FRET efficiency, E_{theo}, is given by numerical integration of $i_{DA}(t)$ and $i_D(t)$

$$E_{theo} = 1 - \int_0^\infty i_{DA}dt \Big/ \int_0^\infty i_D dt \quad (6)$$

By comparing E_{exp} (Eq. (1)) and E_{theo} (Eq. (6)), one can optimize the sole unknown parameter in the latter, the probability of occupation of a given annular site by the acceptor μ (Eq. (3)), which is a direct measure of the protein–acceptor lipid affinity.

3. Results and discussion

The structure of naturally occurring phospholipids found in biomembranes includes phospholipids with mixed acyl chains (one saturated, the other unsaturated at the *sn*-2 position) linked to the glycerol backbone. Such is the case of POPE and POPG, in which a mixture at a molar ratio 3:1 is used to mimic the composition of the *E. coli* inner membrane. Thus, LacY is normally reconstituted into this system or into the total lipid extract of the bacterial membrane where it has been largely demonstrated to be fully functional. In a precedent FRET study [21] delineated to investigate the composition of the annular region of LacY, we have shown that the annulus is apparently enriched in POPE, but that POPG, is also present, depending on bilayer composition. In particular, the results obtained by changing POPE/POPG proportions suggested that the protein can take advantage of a given lipid composition to recruit the most abundant lipid and keep it for its own needs.

The phospholipid composition of the biomembranes, however, varies depending on temperature and growing conditions [40,41]. In physical terms, these changes affect the packing properties of the bilayers. Therefore, to investigate the effects that changes in the *sn*-2-acyl chain can exert on the composition of the annular region of LacY, we have exploited the same FRET strategy followed in the precedent work [21] with phospholipids mixtures of DOPE/POPG, POPE/POPG, and DPPE/POPG all at 3:1 molar ratio. Fig. 2 shows the experimental FRET efficiencies between the single Trp151 of LacY and either Pyr-PG or Pyr-PE as acceptors in the different lipid systems at 37 °C. Fig. 2 also includes the theoretical values calculated from the formalism described in the FRET modelling subsection, using different values of the fitting parameter μ. This parameter represents the probability of finding 0.0 particular phospholipid in the annular region of LacY. The fact that higher FRET efficiencies are obtained for transfer to Pyr-PE (0.232 ± 0.028) compared to Pyr-PG (0.165 ± 0.018) in the POPE/POPG system, and, in a similar way to Pyr-PE (0.231 ± 0.023) compared to Pyr-PG (0.211 ± 0.038) in the DOPE/POPG system, is a first indicator of the selectivity of LacY for PE relative to PG in these compositions. This is confirmed by the quantitative model calculations, which show the best agreement when the experimental values requires an annular region composed of approximately ~90 mol% PE in these systems. Note that 75 mol% would be expected for random distribution of both phospholipids. In the POPE/POPG mixture, the FRET data are compatible with a complete PG exclusion from the annular layer, which is therefore composed solely of PE ($\mu(PE) = 1.00$, $\mu(PG) = 0.00$). Notably, when LacY is reconstituted in DOPE/POPG, the experimental FRET efficiencies indicate an enrichment of PG in the annular region ($\mu(PE) = 0.86$, $\mu(PG) = 0.14$), but still lower concentration than the one expected for uniform lipid distribution. This subtle difference in behaviour cannot be attributed to a possible gel (L_β)/liquid–crystalline (L_α) phase separation. Indeed, the main transition temperature (T_m) of POPE/POPG has been previously established in 20.0 °C [42] and, therefore, at the temperature of the FRET measurements will be in L_α phase. On the other hand, the T_m of the DOPE/POPG mixture is not known, but it is reasonable to assume to be also in L_α phase because both components, DOPE and POPG, present T_m below 37 °C. As a consequence, both mixtures, POPE/POPG and DOPE/POPG, will present uniform distribution of the two components.

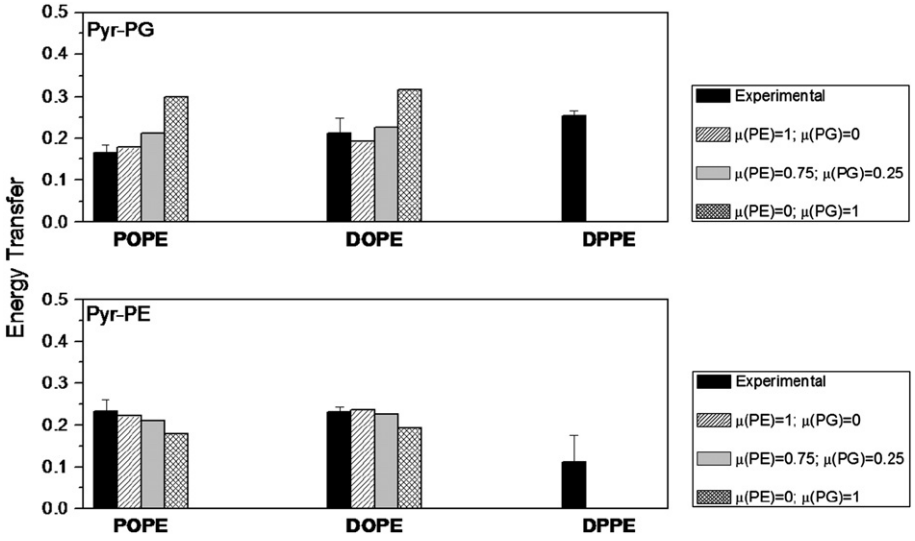

Fig. 2. Comparison of experimental and theoretical values of FRET efficiency between W151 and Pyr-PG (top) and Pyr-PE (bottom) at 37 °C in POPE/POPG (3:1, mol/mol) (left), DOPE/POPG (3:1, mol/mol) (center), and DPPE/POPG (3:1, mol/mol) (right) proteoliposomes (1.5 μM LacY). The error bars stand for $\sigma/\sqrt{n}$, σ being the standard deviation and n the number of measurements performed.

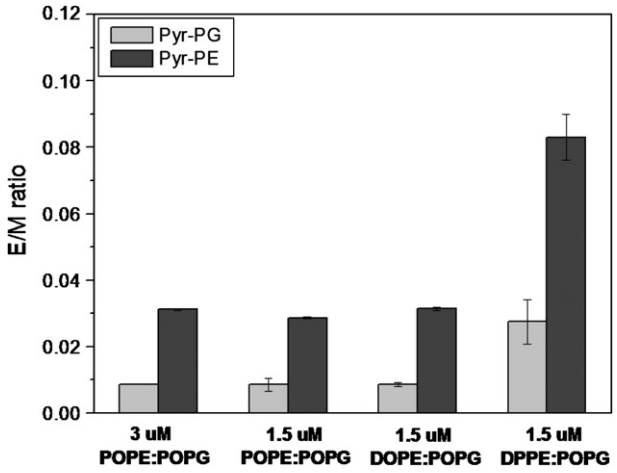

Fig. 3. Excimer-to-monomer ratios (E/M) proteoliposomes of POPE/POPG labelled with directly excited Pyr-PG (grey line) and Pyr-PE (black line) for POPE/POPG (3:1, mol/mol) (3 and 1.5 μM LacY), DOPE/POPG (3:1, mol/mol), and DPPE/POPG (3:1, mol/mol) (1.5 μM LacY). The error bars stand for $\sigma/\sqrt{n}$, σ being the standard deviation and n the number of measurements performed.

Conversely, because the T_m of DPPE is 63 °C [43], L_β/L_α phase separation is expected in the DPPE/POPG mixture, with POPG-enriched fluid domains coexisting with DPPE-enriched gel-phase bilayer regions. The preference of LacY for L_α phases has been verified previously [24,44]. This would imply that, in this lipid composition, LacY is preferably located inside PG-rich or PE-poor domains, which, in turn, would lead to an increase in the efficiency of FRET to Pyr-PG and a decrease in that to Pyr-PE. This variation is indeed verified (Fig. 2), to an extent that the efficiency of FRET to Pyr-PG now clearly surpasses that to Pyr-PE. An accurate application of our FRET model to this biphasic system is not possible at this stage because (i) Pyr-PE and Pyr-PG distribute between the two coexisting phases, according to a partition coefficient, which is unknown, and (ii) even if one assumes that the labelled lipids display the same partition behaviours as their unlabeled counterparts, the phase diagram and hence the phase boundaries, the composition of each phase (most importantly, the amount of residual PE in the PG-rich fluid phase), and the fraction of each phase are not known. Therefore, even if one assumes that all proteins are confined in the fluid domain, and the domains are large enough so that protein donors do not sense acceptors outside them, the local acceptor concentration is still unknown, as also are the relative amounts of DPPE and POPG molecules competing for annular sites with the probe. In any case, these data confirm that LacY shows clear preference for the fluid phase. A simplified application of the model can be attempted, with the following assumptions: (i) all protein is located in the fluid phase, (ii) fluid domains are large in the FRET scale (>5–10 R_0), and (iii) as verified for POPE/POPG, μ(PE) = 1.0, implying that, as long as there is some PE inside the fluid domains, the annular layer will be completely occupied by these molecules. Because the μ parameters have now been fixed, the sole fitting parameters are the acceptor densities in the fluid phase or, to use a reduced, dimensionless variable, the ratios between the acceptor concentration inside the domains and the acceptor concentration for a hypothetical single fluid phase. Fit values of this parameter to the experimental efficiencies are 0.27 for Pyr-PE and 1.56 for Pyr-PG. These are reasonable values (<1 for Pyr-PE and >1 for Pyr-PG), which corroborate the hypotheses of phase separation into PG-rich fluid domains containing LacY and PE-rich protein-depleted gel domains.

When the pyrene-labelled phospholipids are directly excited (338 nm), the excimer-to-monomer (E/M) ratio can provide information on the lateral diffusion and/or local effective pyrene concentration of the labelled phospholipids in bulk. The E/M ratios reported by Pyr-PE and Pyr-PG in the phospholipid mixtures under study are shown in Fig. 3. These ratios were always higher for Pyr-PE than for Pyr-PG, which indicates that Pyr-PE molecules either are globally in a more fluid environment or are mostly distributed with higher local concentration than Pyr-PG. From the FRET experiments, the latter hypothesis is the most likely, as LacY is preferentially surrounded by PE rather than PG. Given the most rigid nature of the annular region, the former hypothesis can be dismissed. The similarity between the values for POPE/POPG and DOPE/POPG agrees with the FRET selectivity data. On the other hand, the introduction of DPPE leads to higher E/M values, which can be attributed to the higher local probe concentration for both, Pyr-PE (in the gel) and Pyr-PG (in the fluid), which results from the coexistence of L_β and L_α phases. For Pyr-PE, the fact that diffusion is considerably slower in the gel phase is probably overturned by formation of static excimers.

In addition to PE and PG, the lipid composition of *E. coli*'s membrane contains 5% to 7% of CL [22,40,41]. CL or diphosphatidylglycerol is a doubly negatively charged four-tailed phospholipid found not only in prokaryotes but also in inner mitochondrial membranes. CL is believed to play a role in membranes that conduct oxidative phosphorylation because it may act as a proton trap [45]. To test the effects of this phospholipid on the annular region, myristoyl-CL and oleoyl-CL were incorporated in the POPE/POPG matrix. We first asked the question if single-W151/C154G LacY is properly folded after reconstitution in these three lipid compositions; so we assayed its binding properties using a modified binding experiment previously used for this mutant [23]. Among all the eight cysteine residues of LacY, there is one, C148, which shows a high reactivity against thiol-reactive agents like maleimides. Notably, C148 modification by some of these reagents is almost avoided when the LacY substrate, TDG is bound to the protein. Therefore, we labeled single-W151/C154G LacY reconstituted in POPE/POPG, POPE/POPG/oleoyl-CL and POPE/POPG/myristoyl-CL vesicles with the fluorescent thiol-modifying reagent fluorescein-5-maleimide in the presence of TDG, L-glucose (negative control) or no substrate, respectively.

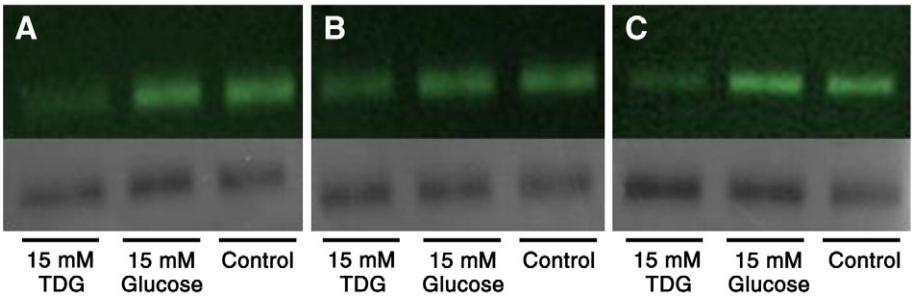

Fig. 4. Substrate recognition by single-W151/C154G LacY reconstituted in proteoliposomes. Fluorescein–maleimide labelling of purified single-W151/C154G LacY reconstituted in vesicles composed by (A) POPE/POPG/oleoyl-CL (67:23:10, mol/mol/mol), (B) POPE/POPG/myristoyl-CL (67:23:10, mol/mol/mol), and (C) POPE/POPG (3:1, mol/mol). As indicated, the experiments were performed in the presence of 15 mM of TDG, 15 mM of L-glucose or no substrate (control). The upper panels (black background) correspond to the fluorescence intensity of fluorescein-labelled protein after being subjected to a 12% SDS–PAGE gel electrophoresis. Lower panels are the same gels after protein staining with Coomassie blue.

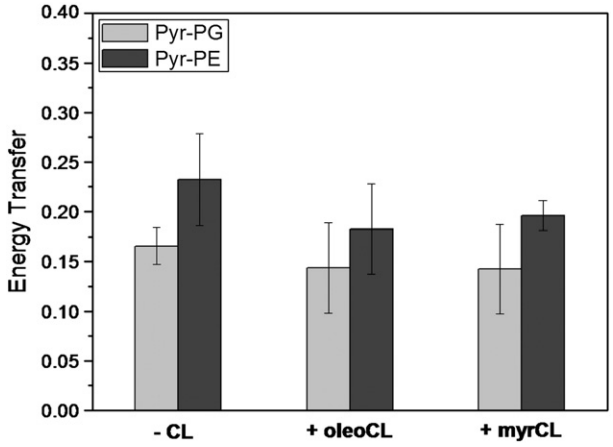

Fig. 5. Experimental values of FRET efficiency between W151 and Pyr-PG (top) and Pyr-PE (bottom) at 37 °C POPE/POPG (3:1, mol/mol) (left), POPE/POPG/oleoyl-CL (0.67:0.23:0.10, mol/mol/mol) (center), and POPE/POPG/myristoyl-CL (0.67:0.23:0.10, mol/mol/mol) proteoliposomes (1.5 μM LacY). The error bars stand for $\sigma/\sqrt{n}$, σ being the standard deviation and n the number of measurements performed.

As seen in Fig. 4, in the three lipid systems and with a slightly different extensions, TDG partially blocks fluorescein labeling, indicating that in all cases the reconstituted protein can selectively recognize the substrate (TDG over L-glucose) and, therefore, is properly folded in this lipid environment.

Notably, the presence of both CL derivatives decreases the efficiencies of FRET when comparing to the same phospholipid mixtures containing no CL (Fig. 5). As can be seen, the FRET values in absence of CL were the highest observed, being 0.165 ± 0.018 and 0.232 ± 0.182 for Pyr-PG and Pyr-PE, respectively. In the presence of CL, the experimental FRET efficiency values, larger for Pyr-PE than for Pyr-PG, suggests that CL displaces POPE and, more extensively, POPG from the annular region of LacY. The fact that the effect is more pronounced for PG than for PE is probably related to the preference of the protein for PE species [12]; see also binary systems above. On the other hand, the effect of oleoyl-CL on LacY-PE energy transfer is slightly more pronounced (0.143 ± 0.045) than that observed for myristoyl-CL (0.142 ± 0.045), suggesting a higher affinity of LacY for the oleoyl derivative. These observations can be, however, rationalized by applying the FRET quantitative model. With a sole exception (see below), significant differences are observed when comparing the experimental FRET efficiencies with the theoretical expectation for random probe distribution, the former being lower. When the acceptor is Pyr-PG, even by imposing segregation of this probe from the first annular layer ($\mu(PG) = 0$), it is still not possible to conciliate the theoretical (0.162) and the experimental values (0.143 for oleoyl-CL, 0.142 for myristoyl-CL), see Table 1. This indicates that, besides being totally excluded from the first layer, PG is also somewhat rarefied beyond it. However, no model refinement was attempted to recover the compositions of both first and second layers (the latter would not be statistically significant). On the other hand, when the acceptor is Pyr-PE, a model matching to the experimental efficiencies (0.183 for oleo CL, 0.196 for myristoyl-CL) requires only partial replacement of PE by CL. When the cardiolipin lipid is oleoyl-CL, the retrieved composition of the annular layer is 40 mol% PE and 60 mol% CL. On the other hand, when the cardiolipin lipid is myristoyl-CL, the composition of the annular layer is 68 mol% PE and 32 mol% CL, indicating that in this case PE is kept in close proximity of the protein, in the same proportion as in the bulk. In the latter case, CL enrichment in the annular layer is solely produced by replacing PG. The fact that myristoyl-CL is not able to displace PE in the same way that oleoyl-CL does is probably due to the hydrophobic mismatch between the short myristoyl acyl chains and the protein.

Table 1
Comparison of experimental and theoretical FRET efficiencies for ternary mixtures PE/PG/CL 67:23:10 at 37 °C.

Lipid composition	Experimental	$\mu(PE) = 0.67$, $\mu(PG) = 0.23$, $\mu(CL) = 0.10$ (all random)	Best fit	Parameter set for composition of first layer
67 POPE/22.75 POPG/10 oleo CL/0.25 Pyr-PG	$E = 0.143$	$E = 0.196$	$E = 0.162$ ($\mu(PG) = 0.00$)	$\mu(PE) = 0.40$, $\mu(PG) = 0.00$, $\mu(CL) = 0.60$
66.75 POPE/23 POPG/10 oleo CL/0.25 Pyr-PE	$E = 0.183$	$E = 0.196$	$E = 0.183$ ($\mu(PE) = 0.40$)	
67 POPE/22.75 POPG/10 myr CL/0.25 Pyr-PG	$E = 0.142$	$E = 0.196$	$E = 0.162$ ($\mu(PG) = 0.00$)	$\mu(PE) = 0.68$, $\mu(PG) = 0.00$, $\mu(CL) = 0.32$
66.75 POPE/23 POPG/10 myr CL/0.25 Pyr-PE	$E = 0.196$	$E = 0.196$	$E = 0.196$ ($\mu(PE) = 0.68$)	

One of the conclusions of this work is the fact that LacY inserts preferentially into the fluid phases of membranes. This is apparently a general principle for which many membrane proteins are excluded from the gel phase [5]. FRET measurements also confirm that PE is the more relevant component of the annular region and that, because it is not displaced by PG or (completely) by CL, it appears to be tightly bounded to LacY. Our results provide evidences on the proximity of PE to the protein (annular layer) under physiologically relevant conditions (*pH*, *T*, and phospholipid composition) providing the structural requirements for protein activity. That is, although it has been demonstrated that binding of H^+ to the Glu 325 is the first step in the putative transport process [46], the origin and maintenance of the electrochemical potential could be related with the ability of PE to provide protons to particular residues of the protein. Similarly to what it has been proposed for another proton depending multidrug transporter LmrP [47] it is likely that PE ineract with Asp-68 which is located in a cytoplasmic loop of LacY and my become the trigger of the transport mechanism. Selectivity of LacY for PE and predominance of this phospholipid at the annular region verified and characterized by FRET measurements and modelling, provide support for a hypothetical coupling between this lipid and LacY during the transport cycle.

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