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Phosphatidylethanolamine–Lactose Permease Interaction: A Comparative Study Based on FRET

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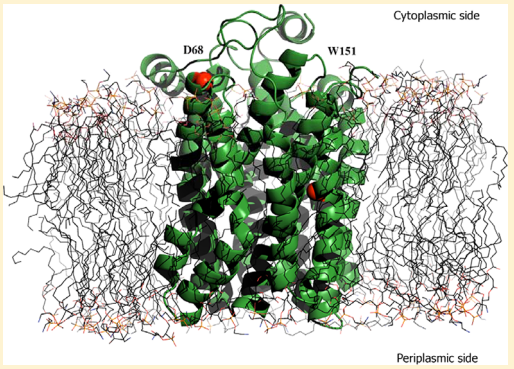
ABSTRACT: In this work we have investigated the selectivity of lactose permease (LacY) of *Escherichia coli* (*E. coli*) for its surrounding phospholipids when reconstituted in binary mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1,2-Palmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-*rac*-(1-glycerol)) (POPG). Förster resonance energy transfer (FRET) measurements have been performed to investigate the selectivity between a single tryptophan mutant of LacY used as donor (D), and two analogues of POPE and POPG labeled with pyrene in the acyl chains (Pyr-PE and Pyr-PG) used as acceptors. As a difference from previous works, now the donor has been single-W151/C154G/D68C LacY. It has been reported that the replacement of the aspartic acid in position 68 by cysteine inhibits active transport in LacY. The objectives of this work were to elucidate the phospholipid composition of the annular region of this mutant and to determine whether the mutation performed, D68C, induced changes in the protein–lipid selectivity. FRET efficiencies for Pyr-PE were always higher than for Pyr-PG. The values of the probability of each site in the annular ring being occupied by a label (μ) were similar at the studied temperatures (24 °C and 37 °C), suggesting that the lipid environment is not significantly affected when increasing the temperature. By comparing the results with those obtained for single-W151/C154G LacY, we observe that the mutation in the 68 residue indeed changes the selectivity of the protein for the phospholipids. This might be probably due to a change in the conformational dynamics of LacY.



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, 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 (TMs) involved in specific transport of molecules across the phospholipid bilayer account for 5–10% and 3% of total proteins encoded by bacterial and human genomes, respectively. Among the secondary transporters, where the source of energy for the process of transport depends on electrochemical potential gradient of ions such as Na^+ or H^+, one of the most studied groups is formed by the 12-TMS family characterized by the presence of 12 transmembrane segments (α-helix).[1,2] Many of these proteins are the therapeutic targets of several drugs and play an important role in conferring drug resistance (to anticancer drugs and antibiotics) in both bacteria and eukaryotic cells. One of the paradigmatic models of the transport protein is lactose permease (LacY) from *Escherichia coli*,[3] the best characterized of all proteins belonging to the 12-TMS group that also includes the LmrP efflux pump in *Lactococcus lactis*[4] or NorA of *Staphylococcus aureus*,[5] that actively expel daunomycin and norfloxacin, respectively. LacY is a lactose cotransporter (symporter) against gradient (uphill) that is coupled with the proton electrochemical potential gradient. The three-dimensional structure of the C154G mutant of LacY[6] and other LacY mutants[7−9] obtained by X-ray diffraction have definitively contributed on the understanding of the mechanism of lactose/H^+ cotransport.[3]

Actually, LacY was the first symporter to be solubilized from membrane, purified to homogeneity,[10] and shown to catalyze all the translocation reactions typical of the transport system in

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vivo.[11] LacY is often reconstituted in native *E. coli* polar phospholipid membrane extracts as well as in binary mixtures of phosphatidylgycerol (PG) and phosphatidylethanolamine (PE) that mimic the inner membrane of the bacteria. The presence of PE is required not only for its function[12] but also for its correct folding in the membrane during biogenesis.[13] Furthermore, the addition of specific phospholipids to the detergent-purified protein has been recognized as the key for obtaining suitable crystals for X-ray diffraction[14] a fact that emphasizes the strong interplay between LacY and phospholipids.

It has been hypothesized[4,15] that the aspartic acid 68 (D68), a highly conserved residue in the major facilitator superfamily (MFS), where LacY belongs, mediates the interaction between PE and transporters of this superfamily. It has been demonstrated that this residue is important for the protein to be sensitive to the proton gradient but also that it plays a role in facilitating conformational changes needed for substrate translocation.[16] In fact, although D68 position is very sensitive to replacement, several second-site activity revertants have been described,[17] which suggests that D68 is not absolutely irreplaceable for the transport mechanism. D68 mutants are still able to bind substrate, but its translocation is locked. On this basis, the proposed mechanism is that the D68 mutation decreases the probability of opening of the hydrophilic pathway on the periplasmic side of LacY upon sugar binding. This is reinforced by the findings that not only the negative charge of the aspartic acid but also the structure of the amino acid is important in this position. In this regard, even the most conservative replacement, D68E, inactivates LacY transport.[18] This is because D68 interacts with K131 forming a weak H bonding pair which enables LacY dynamics. On the contrary, replacement of position 68 with glutamate results in a stronger charge-pair interaction with lysine 131, thus preventing the necessary chain reorganization for sugar-induced opening of the periplasmic cavity. In this regard, Lensink et al.[15] performed LacY molecular dynamics (MD) simulation studies to investigate specific protein−lipid interactions. These authors found only one lipid-mediated salt bridge between conserved residues in LacY, D68 being the crucial amino acid in this interaction. This bond involves two residues, D68 and K69, and a phospholipid, preferably PE over single and double methylated PE. Interestingly, it has been shown that PC interacts also, weaker than the unmethylated PE, and that PG is never involved in the interaction. Importantly, these results are the first to suggest a possible mechanism for the direct interaction between PE and a member of the MFS transporters.

In previous works[19−21] we have exploited Förster resonance energy transfer (FRET) to study lipid selectivity between a single tryptophan mutant of LacY (W151/C154G), used as donor (D), and different phospholipids labeled with pyrene in the acyl chains that are used as acceptors (A). In the present work we have used W151/C154G and delineated a new mutant W151/C154G/D68C, in which the aspartic acid residue has been replaced by cysteine. The strategy consists of measuring the FRET efficiency between the single tryptophan mutants reconstituted in PE:PG 3:1, mol/mol matrixes, and two different acyl-chain pyrene-labeled phospholipids used as acceptors, Pyr-PG or Pyr-PE. The objectives of these experiments are 2-fold: (i) to ascertain the composition of the annular lipids that surround LacY; (ii) to investigate if the D68C mutation will result in changes in such affinity.

2. MATERIALS AND METHODS

N-Dodecyl-β-D-maltoside (DDM) was purchased from Anatrace (Maumee, OH). 1,2-Palmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-dioleoyl-*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) were purchased from Avanti Polar Lipids (Alabaster, AL). 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), and Bio-Beads SM-2 were purchased from Bio-Rad (Hercules, CA). All other common chemicals were ACS grade.

2.1. Bacterial Strains and Protein Purification. Single-151W/C154G/D68C LacY mutant was obtained using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) from *E. coli* BL21(DE3) cells (Novagen, Madison, WI) containing plasmid pCS19 encoding single-W151/C154G LacY donated by Dr. H. Ronald Kaback (UCLA, Los Angeles CA). The resultant plasmid pCS19 encoding single-W151/C154G/D68C LacY construct was confirmed by DNA sequencing. The purification of this mutant was achieved following procedures detailed in previous papers.[19−21] Briefly, *E. coli* was grown in Luria−Bertani broth at 30 °C containing ampicillin (100 μg/mL) and induced at the appropriate moment with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. 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). Protein eluted with 150 mM imidazole was subjected to gel filtration chromatography using a Superdex 200 10/30 column (GE-Healthcare, UK) equilibrated with 20 mM Tris-HCl (pH 7.5), 0.008% DDM. The protein was concentrated using Vivaspin 20 concentrators (30 kDa cutoff; Vivascience, Germany) and stored on ice. Protein identification was performed by SDS/PGE electrophoresis, and protein quantitation was carried out using a micro-BCA kit (Pierce, Rockford, IL).

2.2. Vesicle Preparation and Protein Reconstitution. Liposomes and proteoliposomes were prepared according to methods published elsewhere.[19−21] 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_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 μM). The mole fraction of fluorescent probe (relative to total lipid) 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, and applying successive cycles of freezing and thawing below and above the phase transition of the phospholipids, and sonication for 2 min in a bath sonicator. Afterward, 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

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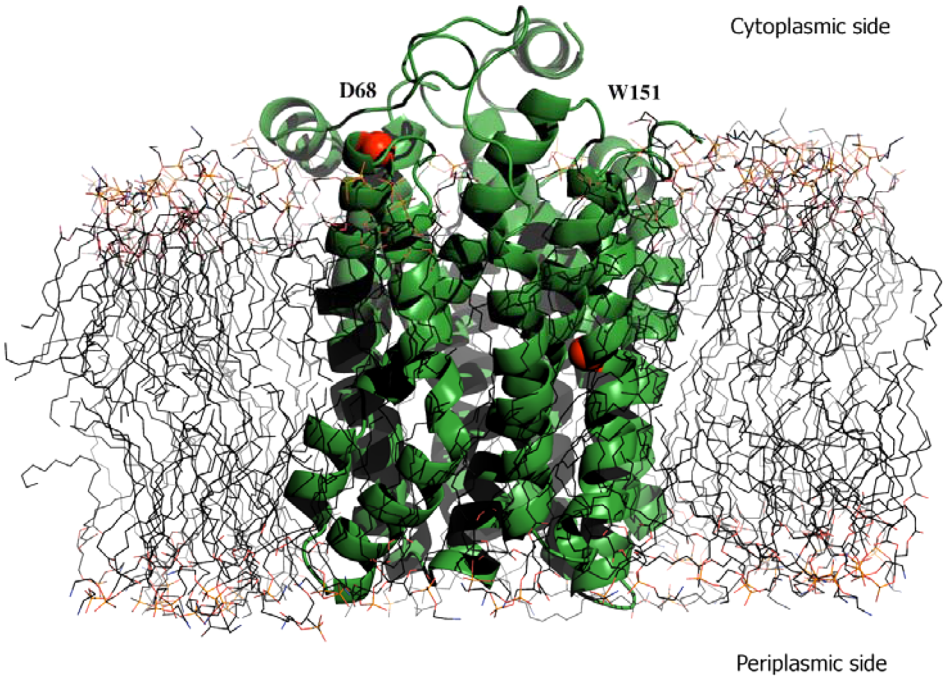

Figure 1. Model of LacY C154G embedded in a POPE matrix. Tryptophan 151 and aspartic acid 68 are highlighted. This model has been constructed using CHARMM-GUI Membrane Builder.

gentle agitation, to obtain a lipid to protein ratio (w/w) of 40. DDM was extracted by addition of polystyrene beads.

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 by adapting a protocol previously described,[20,21] based on the protection of the substrate against thiol modification of LacY. Briefly, 50 μL of proteoliposomes containing 1.5 μM single-W151/C154G/D68C LacY[22] were incubated at room temperature for 5 min with either 15 mM β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (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 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 to the total amount of protein after staining the same gel with Coomassie blue.

2.4. FRET Modeling. Steady-state fluorescence measurements were carried out with an SLM-Aminco 8100 (Urbana, IL) 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 24 °C and 37 °C. The excitation and emission bandwidths were 4/4 and 8/8 nm, respectively. As described in detail elsewhere,[20,21] single tryptophan-LacY mutants (either W151/C154G or W151/C154/D68C), the donors (D), were excited at 295 nm and emission of the pyrene was recovered at 375 nm.

FRET efficiencies (E) are calculated according to the equation

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

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. The description of the model used to fit the data has been clarified in detail elsewhere.[21] Briefly, we assume the existence of two populations of acceptors (A), one located at the annular shell around the protein and another in the bulk outside it. Then we can write the decay of the fluorescence of the donor D as

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

where i_D and i_{DA} are the donor fluorescence decays in the absence and presence of acceptor molecules, respectively. Because the number of annular pyrene phospholipids around each protein molecule is expected to follow a binomial population,[23] the annular contribution to the decay can be expressed as

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

where m is the number of phospholipid molecules in the first layer surrounding the protein and k_t is the rate constant for D−A energy transfer, given by

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

where in turn τ is the donor lifetime and R_0 is the Forster radius (3.0 nm for the Trp/pyrene),[24] and μ is defined as the probability of each site in the annular ring being occupied by a labeled pyrene phospholipid. This probability can be written more intuitively as

$$\mu = K_s\frac{n_{pyr}}{n_{pyr} + n_{PL}} = K_s X_{pyr} \tag{5}$$

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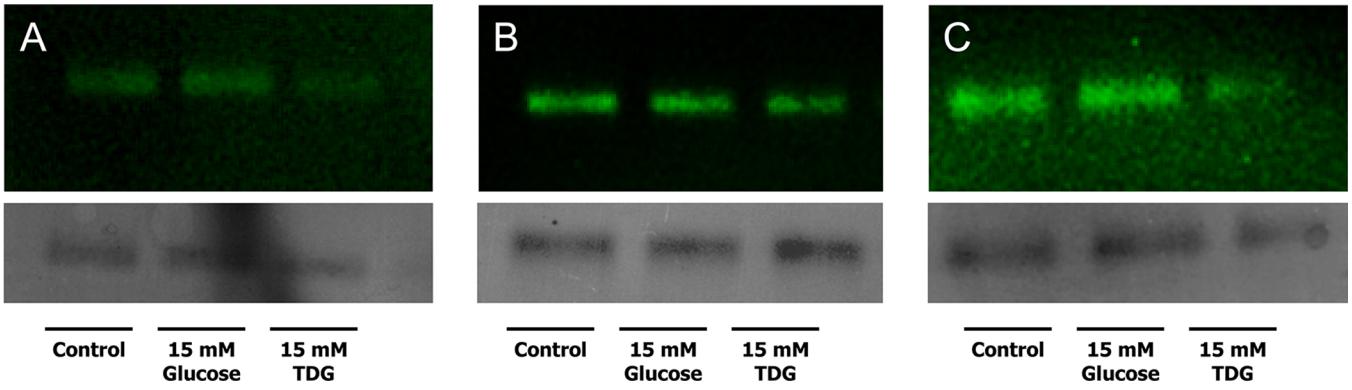

Figure 2. Substrate recognition by single-W151/C154G/D68C LacY reconstituted in DOPE:POPG 3:1 (mol:mol) (A), POPE:POPG 3:1 (mol:mol) (B), and DPPE:POPG 3:1 (mol:mol) (C) proteoliposomes. Fluorescein-maleimide labeling was performed in the presence of 15 mM TDG, 15 mM L-glucose, or no substrate (control). The upper panels (black background) correspond to the fluorescence intensity of fluorescein-labeled protein after being subjected to a 12% SDS−PAGE gel electrophoresis. Lower panels are the same gels after protein staining with Coomassie Blue.

where n are the mole numbers of the labeled (n_{pyr}) and the nonlabeled (n_{PL}) phospholipids, X_{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 than in the bulk, whereas $K_s = 0$ means no acceptor in the annular region. Finally, the rate of FRET to acceptors randomly located outside the annular layer ($\rho_r(t)$) is given by

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

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 acceptors.

RESULTS AND DISCUSSION

A model of LacY embedded in a bilayer constituted by POPE matrix is shown in Figure 1. The position of W151 that acts as a donor for the FRET experiments is highlighted along with the position of D68, located in helix II facing to the cytoplasmic side of the protein. To assess the folding state of the proteins in the proteoliposomes, binding assays have been performed. Figure 2 shows that for all matrix, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) partially blocks fluorescein labeling, indicating that the reconstituted protein can selectively recognize the substrate (TDG over L-glucose) and, therefore, is properly folded in those lipid environments.

A large amount of evidence supports the specific requirement of PE for the proper folding, adequate topology, and activity of LacY.[13,25] Because it is well established that PE may create extensive intermolecular hydrogen bonding, it becomes tentative to hypothesize that a specific interaction between the amine headgroup of PE and some specific amino acid residues of the protein may exist.[4,15] This may be a crucial event that triggers the whole mechanism of active transport. Nevertheless, not only the headgroup is important:[26,27] in recent works where the protein was reconstituted in single phospholipid matrixes[21] and in others based on measuring transport of genetically modified bacteria,[28] the importance of the acyl chain moiety in the interaction between phospholipids and membrane proteins has been pointed out.

The D68 residue, situated at the edge of the interface between helix II and the intracellular loop II-III, is a highly conserved residue in the MFS that has been proposed to mediate the interaction between PE and the protein in the cases of LacY[15] and LmrP,[4] another member of this superfamily. It has been also suggested that this interaction is necessary for the protein to be sensitive to the proton gradient.[4] To further investigate this system, our work's aim is to study if this particular mutation, D68C, would lead to a change of the phospholipid composition intimately interacting with the protein. Particularly, and giving that this mutation inhibits LacY conformational changes after ligand binding,[16] our observations may provide new evidence on the relation between lipid−protein selectivity and conformational mobility of LacY in the membrane.

Figure 3 shows the experimental E values at 25 °C and 37 °C obtained for W151/C154/D68C LacY with both Pyr acyl-labeled phospholipids (Pyr-PE and Pyr-PG) in the three binary phospholipids systems under study (DOPE:POPG, POPE:-POPG, and DPPE:POPG, all at 3:1 molar ratio). Clearly, Pyr-PE is preferred over Pyr-PG, at both temperatures and all phospholipid matrixes. Consequently, the values of μ listed in Table 1 are always the highest for Pyr-PE irrespective of the matrix. These results coincide qualitatively with those observed in POPE:POPG and DOPE:POPG systems, using the W151/C154G mutant of LacY.[20] To rationalize these, we should take in consideration the thermotropic nature of the mixtures used. Thus, while at 25 °C and 37 °C DOPE:POPG remains in the L_α phase and DPPE:POPG exhibits coexistence between the L_α and L_β phases at both temperatures, POPE:POPG still exhibits the L_β phase at 25 °C and L_α phase 37 °C.[29] Because LacY inserts preferentially in fluid phases,[29,30] the negligible variations in the μ values when increasing the temperatures indicate that the lipid environment around W151/C154G/D68C mutant does not change.

For a better comparison Figure 4 shows the normalized values of E between single-W and Pyr-PE (the phospholipid of most interest for these studies) at 37 °C for both LacY versions. As can be seen, the normalized values of E were similar for both mutants in DOPE:POPG, and lower and higher normalized E values were observed in POPE:POPG and DPPE:POPG, respectively, for the systems where the 68 residue has been mutated into cysteine. These results support the preference for

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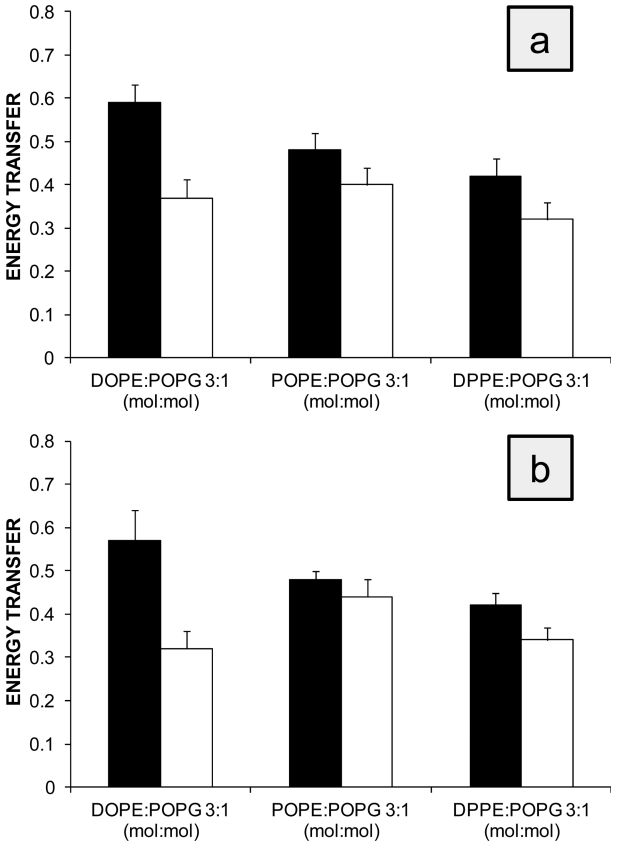

Figure 3. FRET efficiency between W151 of the mutant W151/C154G/D68C LacY and Pyr-PE (black columns) and Pyr-PG (white columns) in different lipid matrixes at 25 °C (a) and 37 °C (b). Proteoliposomes (1.5 μM LacY) of DOPE:POPG 3:1 (mol:mol), POPE:POPG 3:1 (mol:mol), and DPPE:POPG 3:1 (mol:mol) were doped with $x = 0.0025$ of label. The error bars stand for $\sigma/n^{1/2}$, σ being the standard deviation and n the number of measurements performed.

Table 1. Experimental Efficiencies E and Probabilities μ for Each Site in the Single-W/C154G/D68C LacY Annular Ring Being Occupied by a Pyrene Phospholipid

composition (3:1, mol/mol)		experimental E	μ
	25 °C		
DOPE/POPG	Pyr-PE	0.59	0.05
	Pyr-PG	0.37	0.02
POPE/POPG	Pyr-PE	0.48	0.03
	Pyr-PG	0.40	0.02
DPPE/POPG	Pyr-PE	0.42	0.02
	Pyr-PG	0.32	0.01
	37 °C		
DOPE/POPG	Pyr-PE	0.57	0.05
	Pyr-PG	0.32	0.01
POPE/POPG	Pyr-PE	0.48	0.03
	Pyr-PG	0.44	0.03
DPPE/POPG	Pyr-PE	0.42	0.02
	Pyr-PG	0.34	0.01

PE in systems in the L_α phase as well as a higher relative affinity between POPE and W151/C154G than between POPE and W151/C154G/D68C. That is, the introduction of the mutation in the 68 site results in a change of LacY affinity for POPE, leading to a modification in the composition of the phospholipids in close contact with the protein. Interestingly,

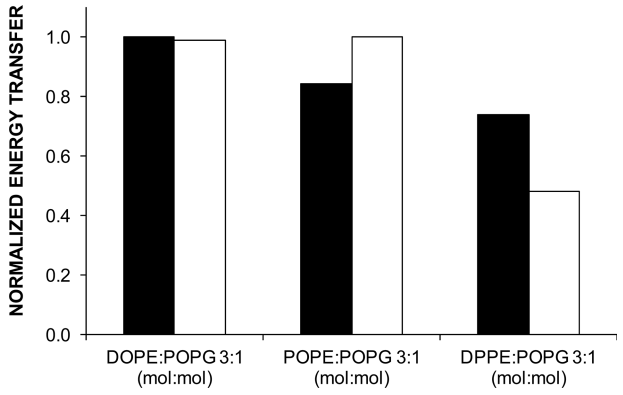

Figure 4. Comparison of normalized FRET efficiency at 37 °C between W151 of the mutant single-W/C154G/D68C LacY and Pyr-PE (black columns) and W151 of the single-W/C154G LacY and Pyr-PE (white columns).

while the model could not be applicable for the DPPE:POPG system when the donor was W151/C154G, efficient energy transfer occurs when using the W151/C154/D68C mutant. Clearly the D68C mutant studied here is able to recruit Pyr-PE to its vicinity even in the presence of phase separation. This is at variance with the W151/C154G. In that case, Pyr-PG presented more efficient FRET values than Pyr-PE in DPPE:POPG as a consequence of L_α and L_β coexistence.[20]

These observations are in concordance with the earlier finding that LacY promotes phospholipid microdomain formation around the protein.[31] The evidence of such a laterally segregated domain was gathered from two types of monomer to excimer ratio measurements: among pyrene-labeled phospholipids and between pyrene-labeled LacY and surrounding pyrene-labeled phospholipids. Although the interpretation was based on the mismatch between the protein and the phospholipids, our results show that the lipid selectivity depends on a complex mechanism of phospholipids adaptation to the protein surface,[26,27] that should be related to the lateral pressure profile of each species[32] and/or intrinsic curvature of the phospholipids.[33] According to MD simulations,[15] however, the specific interaction between PE and aspartic acid 68 could be anticipated. Hence, this mutation (D68C) may hinder the PE-68 residue interaction and consequently modify the protein−lipid affinity.

In conclusion, our results suggest that the mutation in the 68 position positively changes the selectivity of the protein for phospholipids and may induce a change in the conformational dynamics of LacY by recruitment of the phospholipid species most adaptable to the geometrical needs of the protein. Thus, while the lipid selectivity for D68 is DOPE ~ POPE > DPPE, the mutation increases the protein selectivity for DOPE over POPE and DPPE. This observation demonstrates the implication of the acyl chains in the interaction and points to a possible requirement for the heteroacid phospholipids besides the headgroup requirement. Furthermore, it is demonstrated here that although POPE is still present in the boundary of W151/C154G/D68C LacY, the mutation decreases the preference of the protein for this phospholipid. This might support the proposed mechanism that this amino acid is involved in the interaction between the protein and POPE,[4] possibly forming a lipid-mediated salt bridge,[15] an event that can be intimately related with the increase of the open

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probability of the periplasmic side of LacY upon sugar binding.[16]

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Notes

The authors declare no competing financial interest.

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