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Effects of lactose permease of *Escherichia coli* on the anisotropy and electrostatic surface potential of liposomes

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Abstract

The membrane transport protein lactose permease (LacY), a member of the Major Facilitator Superfamily (MFS) containing twelve membrane-spanning segments connected by hydrophilic loops, was reconstituted in liposomes of: (i) 1,2-dimyristoyl-*sn*-glycero-3-phosphocoline (DMPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in equimolar proportions; and (ii) *Escherichia coli* total lipid extract. The structural order of the lipid membranes, in the presence and absence of LacY, was investigated using steady-state fluorescence anisotropy. The features of the anisotropy curves obtained with 1,6-phenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluene sulfonate (TMA-DPH) evidenced: (i) the insertion of LacY into the bilayer; and (ii) a surface effect on the membranes. The most dramatic effects were observed when LacY was reconstituted in the *E. coli* lipid matrix. The effect of the protein on the electrostatic surface potential of each bilayer was also examined using a fluorescent pH indicator, 4-Heptadecyl-7-hydroxycoumarin (HHC). Changes in surface potential were enhanced in the presence of the substrate (i.e. lactose) only when the lipid matrices were charged. These results suggest a role for charged phospholipids (i.e. phosphatidylethanolamine or phosphatidylglycerol) in proton transfer to the amino acids involved in substrate translocation.

1. Introduction

Membrane proteins constitute 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 may be involved in transport functions. Among them, lactose permease (LacY), one of the most widely studied cytoplasmic membrane proteins, is often taken as a paradigm for secondary transport proteins that couple the energy stored in an electrochemical ion gradient to a concentration gradient (β-galactoside/H^+ symport). LacY belongs to the Major Facilitator Superfamily (MFS) [1], most of whose members are predicted to contain twelve transmembrane segments. After years of tedious trial-and-error experiments a three-dimensional (3D) model obtained from X-ray diffraction studies is now available [2]. LacY consists of twelve transmembrane α-helices, crossing the membrane in a zig-zag fashion, that are connected by eleven relatively hydrophilic, periplasmic and cytoplasmic loops, with both amino and carboxyl termini on the cytoplasmic surface. In Fig. 1 we show different views of the surface model and the electrostatic potential rendered by using the program Protein Explorer [3]. Two-dimensional crystallization was earlier achieved [4], and we have recently published a preliminary atomic microscopy study of those 2D crystals [5] that offers excellent opportunities for protein manipulation.

In this study the effects of LacY on the structural order of lipid membranes was investigated by measuring, as a function of temperature, the steady-state fluorescence anisotropy (r_s) of TMA-DPH and DPH incorporated into liposomes and proteo-liposomes of LacY with neutral (DMPC : POPC, 1 : 1, mol/mol) and charged *E. coli* total lipid extract phospholipids as convenient phospholipid matrices. It is worth noting that DPH is embedded in the bilayer while TMA-DPH remains anchored at the aqueous interface of the phospholipid bilayer, its DPH moiety being intercalated between the phospholipid acyl chains. Hence, by using both probes, we are able to obtain information on the effects caused by the integration of LacY

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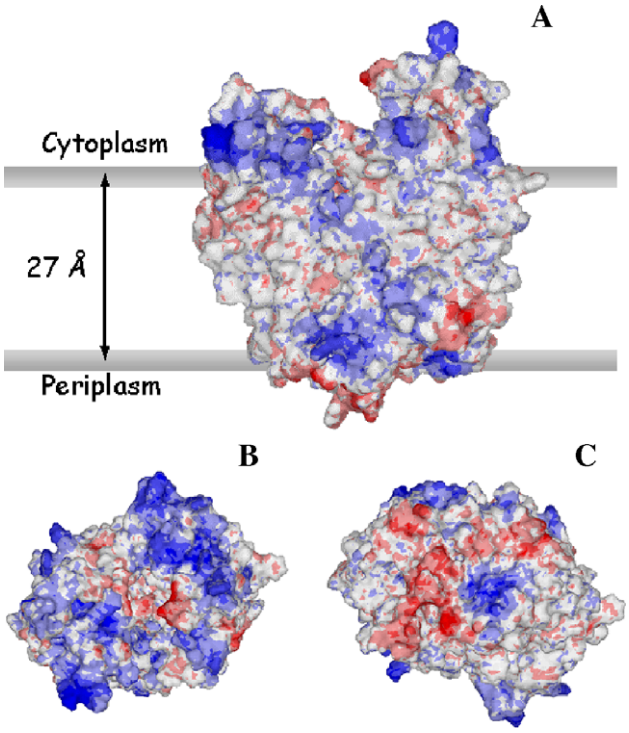

Fig. 1. The surface model and electrostatic potential of lactose permease of *E. coli*, calculated with the program Protein Explorer [3]. The polar surfaces are colored blue (positively charged) and red (negatively charged). View parallel to the membrane (A), and along the membrane normal from the cytoplasmic (B) and periplasmic (C) side.

into the core and at the interface regions of the bilayer. When a transmembrane protein is reconstituted into liposomes it may affect the electrostatic surface potential of the membranes. Thus, the protein contribution to the membrane electrostatic surface potential can be assessed by using a fluorescent pH indicator, 4-Heptadecyl-7-hydroxycoumarin (HHC) [6], and this has been extensively used in determining the electrostatic surface potential of monolayers [7] and liposomes [8]. Its fluorometric titration, when incorporated in liposomes, facilitates determination of the interfacial pK of the probe and calculation of the variation of the electrostatic surface potential ($\Delta\psi$) [9] after reconstitution of LacY in the same phospholipid matrix. This information could be of considerable interest in establishing a possible relationship between the electrical properties of the phospholipid bilayer surface and LacY activity [10].

2. Materials and methods

2.1. Chemicals

1,2-dimyristoyl-*sn*-glycero-3-phosphocoline (DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and *E. coli* total lipid extract, acetone/ether washed phospholipids (nominally 57.5% phosphatidylethanolamine, 15.1% phosphatidylglycerol, 9.8% cardiolipin, 17.6% others) were purchased from Avanti Polar Lipids (Alabaster, AL). The probes 1,6-phenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluene sulfonate (TMA-DPH) and 4-Heptadecyl-7-hydroxycoumarin (HHC) were obtained from Molecular Probes (Eugene, OR). *N*-dodecyl-β-D-maltoside (DDM) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) and isopropyl-1-thio-β, D-galactopyranoside (IPTG) were purchased from Ecogen (Barcelona, Spain), and Bio-Beads SM-2 was purchased from Bio-Rad (Hercules, CA, USA). All other common chemicals were ACS grade.

2.2. Bacterial strains and protein purification

E. coli T-184 cells [$lacI^{+}O^{+}Z^{-}$*(A), rpsL, met, thr, recA, hsdN, hsdR/F,* $lacI^{q}O^{+}Z^{D118}$ *(*$Y^{+}A^{+}$*)*] were kindly provided by Dr. H. Ronald Kaback of HHMI-UCLA. LacY was extracted and purified from the overproducing strain *E. coli* T-184. Briefly, cells were grown aerobically at 37 °C in Luria–Bertani broth containing ampicillin (100 μg mL^{-1}) and streptomycin (10 μg mL^{-1}). Dense cultures were diluted in a 40 L fermentor and grown for 1 h at 37 °C before induction with 0.3 mM IPTG. After growing for another 3 h at constant temperature, the cells were harvested and disrupted by passage through a French press. The membrane fraction was isolated by centrifugation and extracted with 2% (w/v) DDM and LacY purified by affinity chromatography on immobilized monomeric avidin beads, as described previously [5,11]. The column was washed with approximately 200 mL 50 mM NaP_i, 150 mM NaCl, pH 7.50, 0.02% DDM and the permease eluted with 2 mM biotin in the same buffer, concentrated and dialyzed using a Micro-ProDiCon membrane (Spectrum). The protein was quantified using the spectrophotometric assay Micro-BSA (Pierce).

2.3. Vesicle preparation and protein reconstitution

Chloroform/methanol (50:50, v/v) solutions containing the appropriate amounts of phospholipids were dried under a stream of oxygen-free N_2 in a conical tube. The thin film obtained was kept under high vacuum for approximately 3 h to remove organic solvent traces. Multilamellar liposomes were obtained after redispersion of the film in 50 mM KP_i buffer, pH 7.50 and sonication for 30 min in a bath sonicator [11]. The liposomes were then dissolved in DDM and mixed with the solubilized protein to a final lipid-to-protein ratio (LPR) of 50:1 (w/w) and incubated at 4 °C for 30 min with gentle agitation. Extraction of DDM was achieved by addition of polystyrene beads (Bio-Beads SM-2, Bio-Rad). The first two extractions were performed at room temperature for 2 h each, while the last extraction was performed at 4 °C overnight. The vesicles were collected by centrifugation for 4 h at 35,000 rpm and resuspended in a small volume of 50 mM KP_i pH 7.50, followed by three cycles of freezing and thawing. The samples were frozen and stored in liquid nitrogen at a final protein concentration of 1 mg/mL. Immediately before use the samples were subjected to freeze/thaw and brief periods of sonication in order to obtain unilamellar vesicles. Samples were kept at room temperature prior to fluorescence experiments.

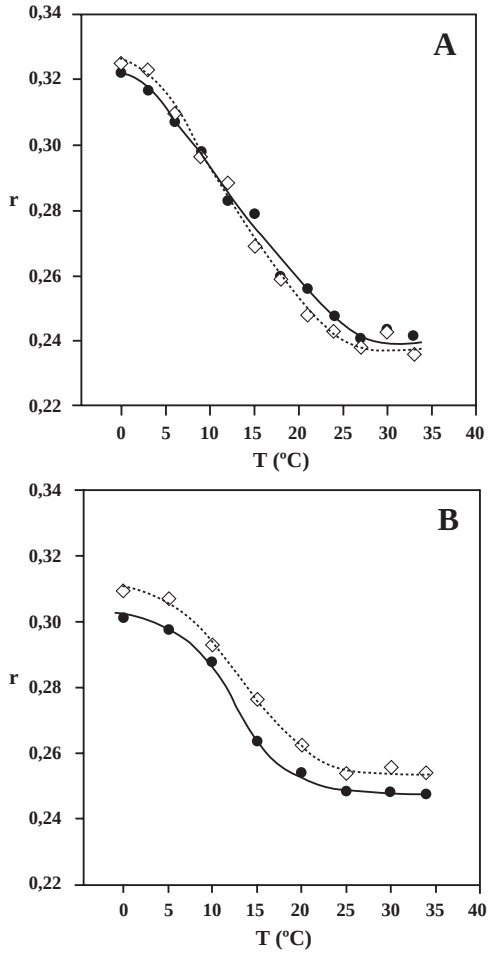

Fig. 2. Steady-state polarization of TMA-DPH for vesicles formed with (A) DMPC:POPC (1:1, mol/mol), and (B) *E. coli* lipid extract liposomes. Liposomes (filled circles) and proteoliposomes of LacY (open diamonds) are shown. Each point indicates the mean value of three experiments. Data were fitted to sigmoid curves as described in the Methods section.

2.4. Steady state anisotropy experiments

The experimental procedures to measure the steady-state anisotropy of TMA-DPH and DPH were adapted from previous stud [12]. Essentially, 3 μl of concentrated stock solution of either probe in methanol was added to 1500 μl of vesicle (liposome or proteoliposome) suspension for 30 min at 37 °C. The final lipid/fluorescent probe ratio was 1000:1; mol/mol. The excitation wavelength was 381 nm and emission was measured at 426 nm. The anisotropy was recorded at 3° intervals in the range 0 to 35 °C. The vertically and horizontally polarized emission intensities were corrected for background scattering by subtraction of the corresponding polarized intensities of a blank containing the unlabelled suspension. Steady-state anisotropy (r_s) values were calculated according to

$$r_s = \frac{I_{VV} - GI_{HV}}{I_{VV} + 2GI_{HV}} \quad (1)$$

where I_{VV} and I_{HV} are the intensities measured in directions parallel and perpendicular to the exciting beam, respectively, and G is the grating correction factor equal to I_{HV}/I_{HH}. The anisotropy values shown in Figs. 2 and 3 are the average of several measurements. The individual points were within 5% of the reported values.

The following equation was fitted to the anisotropy versus temperature data

$$r_s = r_{s2} + \frac{r_{s1} - r_{s2}}{1 + 10^{B'\left(\frac{T}{T_m} - 1\right)}} \quad (2)$$

where T is the absolute temperature, T_m is the midpoint phase transition and r_{s1} and r_{s2} are the upper and lower values of r_s; B' is the slope factor which is correlated with the extent of cooperativity (B) by $B = [1 - 1/(1 + B')]$; the introduction of B yields a convenient scale of cooperativity ranging from 0 to 1.

2.5. Measurement of the absolute surface potential of charged liposomes

The variation in the electrostatic membrane potential ($\Delta\psi$) was calculated in triplicate, adapting a method used elsewhere [9,12]. Briefly, in these experiments the molar ratio of total lipid to fluorescent probe was 1000:1. Similar molar ratios and

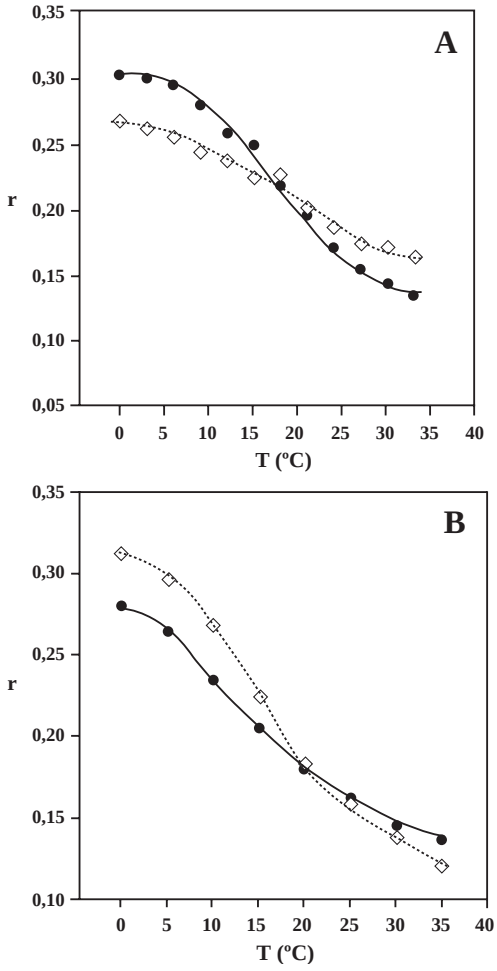

Fig. 3. Steady-state polarization of DPH for vesicles formed with (A) DMPC:POPC (1:1, mol/mol), and (B) *E. coli* lipid extract liposomes. Liposomes (filled circles) and proteoliposomes of LacY (open diamonds) are shown. Each point indicates the mean value of three experiments. Data were fitted to sigmoid curves as described in the Methods section.

ionic strength were maintained in the experiments carried out in the presence of LacY in order to calculate ΔpK in the presence of liposomes. Fluorescent probe was incorporated into the bilayer by bath incubation at 37 °C for 30 min. Samples were kept at room temperature for another 30 min to achieve thermal stabilization before measurements were performed. The electrostatic surface potential was then calculated using

$$\Delta\Psi = \frac{-2.3RT}{F}(\Delta pK_0 - \Delta pK) \quad (3)$$

where ΔpK_0 and ΔpK are the differences between the pK determined in neutral liposome or in the charged liposome system, and the pK of the probe in solution, respectively. When the protein is present, ΔpK_0 and ΔpK are the differences between the pK determined in the liposome system, in the absence or presence of LacY, and the pK of the probe in solution. R is the universal gas constant, T is the temperature and F is Faraday's constant.

3. Results

The temperature dependence of TMA-DPH fluorescence anisotropy in liposomes and proteoliposomes is shown in Fig. 2. For DMPC:POPC (1:1, mol/mol) liposomes (Fig. 2A), in the absence of LacY, the temperature increase leads to a monotonic decrease in r_s values. As can be seen the features of the phospholipid transition were almost unaffected by the presence of LacY. In contrast, a strong effect was exerted by LacY on *E. coli* liposomes (Fig. 2B). In this case, for the entire range of temperatures studied, anisotropy values were lower in liposomes than in proteoliposomes. The increase in r_s values reflects an increase in the molecular order at the interface region due to the presence of LacY. As can be seen in Table 1, the protein induces slight but not significant increases in the T_m of both compositions, whereas the values of B were only moderately affected in the case of *E. coli* liposomes.

In the experiments with DPH (Fig. 3) the temperature profiles of r_s for liposomes and proteoliposomes were substantially different (Table 2). By comparison with the r_s values in Fig. 2, it can be seen that the anisotropy of DPH dropped to lower values than those observed with TMA-DPH for the same temperature range. Thus, the anisotropy of TMA-DPH dropped from 0.32 to 0.24 when the temperature increased from 0 to 35 °C, whilst that of DPH dropped from 0.30 to 0.13 for the same temperature range. As can be seen in Fig. 3A, for DMPC:POPC (1:1, mol/mol) liposomes, r_s decreased slightly with increasing temperature. Interestingly, whereas LacY diminished the structural order of the bilayer below the main transition, at temperatures above T_m the protein made the fluid phase more ordered. The protein also caused a significant effect on the lipid membranes of *E. coli* (Fig. 3B), increasing and decreasing the anisotropy values, below and above the main phospholipid phase transition, respectively. In this case, however, the decreases of r_s values are almost continuous, the transition becoming a little sharper for the proteoliposomes. According to DPH, the presence of LacY induces a slight decrease of 0.3 °C in T_m for DMPC:POPC (1:1, mol/mol) liposomes and a very small decrease in B (Table 1). In contrast, the presence of LacY induces an increase of approximately 2 °C for the *E. coli* lipid matrix and an increase of 0.14 units in B.

The surface pKs and electrical surface potentials ($\Delta\Psi$) calculated according to Eq. (3) are shown in Table 3. The reference surface potential (0 mV) was assigned to the neutral phospholipid (POPC). The same value, as expected, was obtained for DMPC:POPC (1:1, mol/mol) liposomes. As can be seen in Table 3, the values obtained for the proteoliposomes were positive. As expected from its nominal composition (see Materials and methods), a negative potential was obtained for the *E. coli* liposomes extract (−26 mV). As can be seen in Table 3, the proteoliposomes exhibited positive electrostatic surface potentials for both compositions, the more dramatic change occurring for the *E. coli* proteoliposomes.

4. Discussion

LacY is an extremely flexible protein with 70% of its amino acid residues located in hydrophobic domains [2]. Accordingly, the integration of LacY into liposomes clearly affects the inner core of the bilayer of both phospholipid compositions [13], and this is what DPH experiments reflect. Moreover, TMA-DPH anisotropy experiments have shown a significant effect at the interfacial region of *E. coli* liposomes, and also, to a lesser degree, at the interfacial region of DMPC:POPC liposomes. Despite the differences in acyl chain length, both phospholipids may complement the apolar surfaces of the integral protein. Furthermore, we have shown elsewhere that DMPC and POPC are ideally mixed in monolayers and form self-assembled structures that integrate the protein [14]. It is worth noting that during the reconstitution process, temperatures below (4 °C)

Table 1
Transition temperature (T_m) and cooperativity (B) values obtained by the non-linear adjustment of steady-state polarization data from Fig. 2

	Liposomes		Proteoliposomes of LacY	
	T_m (°C)	B	T_m (°C)	B
DMPC:POPC (1:1)	11.6±0.6	0.48	12.1±0.6	0.51
E. coli lipid extract	12.8±0.3	0.66	13.1±0.5	0.60

Table 2
Transition temperature (T_m) and cooperativity (B) values obtained by the non-linear adjustment of steady-state polarization data from Fig. 3

	Liposomes		Proteoliposomes of LacY	
	T_m (°C)	B	T_m (°C)	B
DMPC:POPC (1:1)	18.0±0.4	0.57	17.7±0.8	0.50
E. coli lipid extract	12.7±0.9	0.40	14.6±0.2	0.54

Table 3
Experimental electrostatic surface potentials calculated using Eq. (3)

	Liposomes		Proteoliposomes of LacY	
	pK	$\Delta\psi$ (mV)	pK	$\Delta\psi$ (mV)
POPC	8,13	0		
DMPC:POPC (1:1)	8,15	0	7.36	+49±8
E. coli lipid extract	8,56	−26±6	6.92	+101±14

and above (room temperature) the transition temperature of the phospholipid mixture are alternated to guarantee the insertion of LacY. This provides an indirect explanation of the suitability of this phospholipid matrix for the immobilization [11] and crystallization of LacY in two dimensions [4,5]. To date, such a goal has yet to be achieved by using *E. coli* lipid extract or another phospholipid composition [12].

LacY has, necessarily, been randomly orientated when it has been reconstituted in liposomes. For this reason the extra- (Fig. 1B) or intracellular (Fig. 1C) loops connecting the twelve transmembrane helices remain exposed to either the inner or outer monolayer of the bilayer [15]. However, a significant number of the residues exposed exhibited positive charge at physiological pH (see coloration in Fig. 1). This results in positive values of the calculated $\Delta\Psi$ for the proteoliposomes. Moreover, the slight variations of T_m induced by LacY in the liposomes suggest that an electrostatic interaction cannot be excluded, in addition to the postulated hydrogen bonds formed between the headgroups of the phospholipids and some of the charged residues in the interfacial loops [10,12]. Importantly, changes in electrostatic surface potential after addition of the substrate (either lactose or TDG) produced changes only in the liposomes of *E. coli* (data not shown). This illustrates that when LacY is embedded in the neutral matrix (DMPC : POPC) the protein is not active.

The changes in the electrostatic potential may therefore indicate a possible coupling between the charged residues in the loops (see Fig. 1) and the molecules of phosphatidylethanolamine and/or phosphatidylglycerol. It is not clear, however, how the proton might reach the residues involved in the proton translocation (Glu325 and Asp 309) [16,17] that are located deeper in the helices. Further studies into the interaction between pyrene phospholipid analogs and single-*Trp* mutants of LacY currently being conducted in our laboratory will likely broaden our understanding of the coupling mechanism between the integral membrane protein and the phospholipids that constitute its immediate vicinity, and which are known as boundary or annular lipids.

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