APÉNDICE

case, other strategies could be considered. Among them crystallization in two-dimensions (2D) [10] or in cubo crystallization [11] have been successfully applied in many cases. To obtain 2D membrane protein crystals, proteolipid sheets (PLSs) were obtained from protein reconstituted in lipid bilayers, normally spread onto an adequate surface (i.e. mica) and observed generally by electron microscopy and more recently by atomic force microscopy (AFM).

The present paper has the following objectives: (i) to demonstrate the expression and activity of Omp1 following the accumulation of a 6-fluoroquinolone antibiotic (6-ciprofloxacin) into the *E. coli* UH302 and *E. coli* UH302 pOM100 cells; (ii) to investigate if ubiquous crystallization of the protein occur by observation in situ of outer membranes extracted from the recombinant *E. coli* UH302 pOM100; and (iii) to select the adequate phospholipid composition to obtain 2D crystals from PLSs. This preliminary work becomes necessary because the phospholipid matrix might eventually determine the protein packing [12]. Besides, it compromise future structural studies on the 2D crystallized membrane protein.

2. Materials and methods

2.1. Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), octyl glucoside (OG), and lipopolysaccharide from *E. coli* serotype Oss:B5 were purchased from Sigma, St. Louis, MO, USA. Genapol was from Fluka, Spain. All other common chemicals were ACS grade.

2.2. Bacterial strains and growth conditions

S. marcescens 2170 is an environmental isolate [13] and *E. coli* UH302 [14], a porin-deficient strain, was used for cloning and expression experiments. Strains were grown in Trypticase Soy Broth (TSB) for outer membrane protein preparations and porin purification, purchased from Liofilchem Bacteriological Products (Italy). Ciprofloxacin (Cip) was kindly supplied by CENAVISA Laboratories (Reus, Spain).

2.3. Expression and purification of the cloned porin in E. coli UH302

The *S. marcescens* Omp1 porin was expressed in *E. coli* UH302. The Omp1 protein was purified as follows: bacteria were growth overnight in TSB supplemented with ampicillin 100 μg/ml and harvested by centrifugation (1000×*g*, 10 min), washed once with a 10 mM Tris–HCl pH 7.4 and resuspended in the same buffer. Cells were broken by ultrasonic treatment and unbroken cells removed by centrifugation (1000×*g*, 10 min). The supernatant was centrifuged (100,000×*g* for 1 h), sedimented bacterial envelopes were resuspended in a buffer containing 2% SDS, 10 mM Tris–HCl pH 7.4. The peptidoglycan layer and the associated proteins were pelleted by centrifugation at 100,000×*g* for 30 min. The pellet was subjected to a second SDS wash. The final pellet was suspended in a buffer containing 2% Genapol, 10 mM Tris–HCl pH 7.4 and 2 mM ethylenediamine tetraacetic acid (EDTA). The supernatant of the subsequent centrifugation (100,000×*g*, 30 min) contained pure Omp1 porin. To visualize Omp fractions, SDS-PAGE was performed in a Bio-Rad apparatus (miniprotean II) gels were stained with 0.25% Coomassie brilliant blue, destained and finally dried using a gel dryer (BioRad 543).

2.4. Determination of fluoroquinolone accumulation

Fluoroquinolone accumulation was followed using a procedure elsewhere described [6] which takes the advantage of the intrinsic fluorescence of the antibiotic [15]. Isolates were incubated at 37 °C until A_{600nm}=0.5–0.7. Bacteria were harvested by centrifugation (9000×*g*) at room temperature, washed and concentrated 10-fold in phosphate buffer saline (PBS) pH 7.5. Fluoroquinolone was added to 1-ml aliquots to a final concentration of 10 μg/ml. At time intervals of 0.25, 0.5, 1.5, 3, 6, 8, 10, 15 and 20 min, samples were centrifuged in a microfuge at 10,000 rpm at 4 °C for 1 min. Pellets were resuspended in 1 ml of 0.1 M glycine–HCl buffer at pH 3.0, and finally incubated at room temperature overnight to allow bacterial lysis. Thereafter, the suspensions were centrifuged at 20 °C for 25 min to remove bacterial debris.

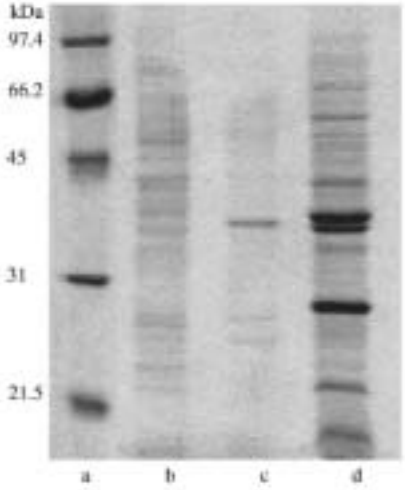

Fig. 1. SDS-PAGE of outer membrane proteins. Standard molecular weight (a), *E. coli* UH302 (b), *E. coli* UH302 pOM100 (c) and *S. marcescens* (d). Omp1 porin is expressed in recombinant strain.

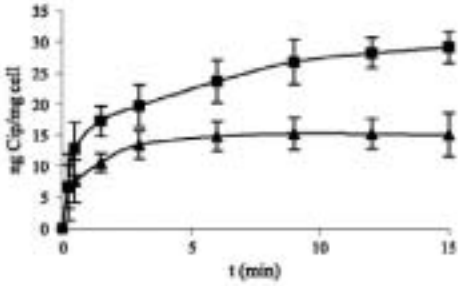

Fig. 2. Ciprofloxacin accumulation of *E. coli* UH302 (▲) and *E. coli* UH302 pOM100. (■) The values are the average of three independent measurements.

The concentration of antibiotic in the supernatants was determined fluorometrically using an SLM Aminco 8100 spectrofluorometer.

2.5. Membrane preparation and protein reconstitution

For protein reconstitution a chloroform/methanol (50:50, v/v) solution of POPC was dried under a stream of oxygen-free N_2 in a conical tube and the thin film obtained kept under high vacuum for approx. 3 h to remove organic solvent traces. Liposomes were obtained by redispersion of the lipid film in 10 mM Tris–HCl, 150 mM NaCl buffer (pH 7.40) applying successive cycles of freezing and thawing, below and above the phase transition of the phospholipid. Thereafter liposomes, supplemented with 2% (w/v) of OG, were sonicated for 30 min in a bath. Purified Omp1 solubilized in OG were mixed with the liposomes to obtain a lipid-to-protein ratio (LPR) (w/w) lower than 1 to a total protein concentration of 100 μM. Several steps have been followed to extract the detergent: (i) incubation of the mixture at 4 °C for 30 min; (ii) incorporation of the sample into dialysis cassette (Slide-A-Lyzer®) in presence of dialysis buffer (10 mM Tris–HCl, 150 mM NaCl pH 7.40); (iii)

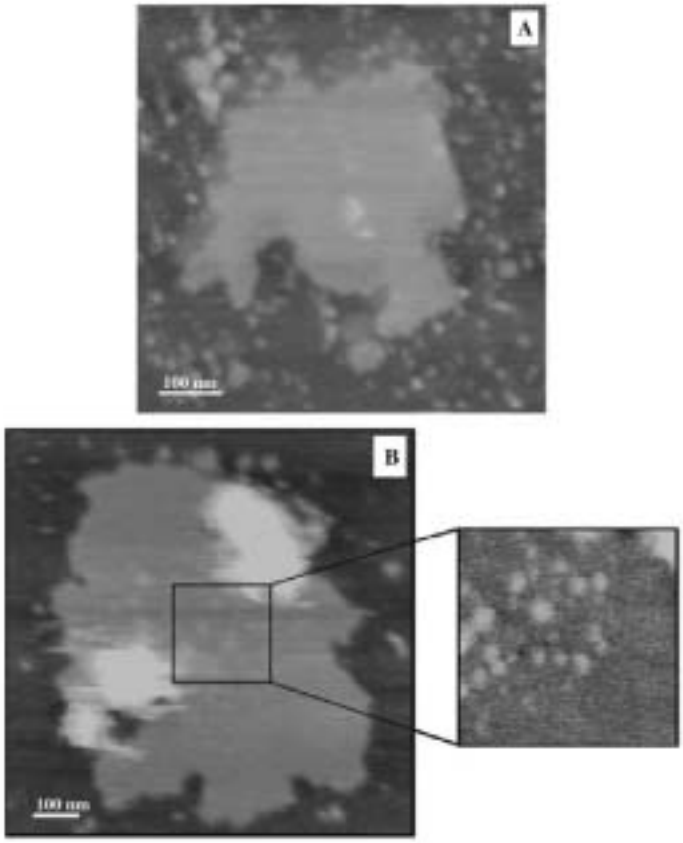

Fig. 3. Atomic force image of topography (tapping mode) of the membrane extracted from *E. coli* UH302 (A) and *E.coli* UH302 pOM100 (B) in 10 mM Tris–HCl pH 7.40, 150 mM NaCl. Zoom of surface bilayer to appreciate a detail of protrusions.

dialysis of the sample during 3 days at room temperature changing the dialysis buffer every 12 hours; (iv) centrifugation at 12,000 r.p.m. for 1 h; (v) resuspension of the pellet to a final concentration 100 μM in buffer 20 mM Hepes, 300 mM KCl, 25 mM $MgCl_2$ pH 6.40; and (vi) stabilization of the sample overnight at room temperature.

2.6. AFM observations

Experiments were carried out as in previous works [16]. Images were recorded in contact or tapping mode with a commercial Digital Instruments (Santa Barbara, CA) Nanoscope III AFM fitted with a 15-μm scanner (d-scanner). Standard Si_3N_4 tips, with a nominal force constant of 0.12 $N \cdot m^{-1}$ (Digital Instruments), were used and the forces exerted by the tip were minimized by previously recording force plots to each sample. Images were obtained in situ using an AFM fitted with a tapping or contact-mode liquid cell. Before every sample, the AFM liquid cell was washed with ethanol and ultra pure water (Milli Q reverse osmosis system), and allowed to dry in a N_2 stream. Mica discs (green muscovite mica) were cleaved with scotch and glued onto a Teflon disc by a water-insoluble epoxy. These Teflon discs were glued onto a steel disc and then mounted onto the piezoelectric scanner. Previous to imaging the sample, the tip-sample pair was thermally stabilized. Then, aliquots of 50 μl of reconstituted Omp1 in proteoliposomes samples were pipetted onto freshly cleaved mica, allowing the sample to stabilize at 25 °C (above the transition temperature of the phospholipid mixture) for 30 min (approx.) and thereafter rinsing the surface with divalent cations free. The tip was immediately immersed in the buffer. To accomplish all these experiments it was necessary to drift equilibrate the cantilever for 30 min before imaging.

3. Results and discussion

Omp1 has been described as a porin throughout small hydrophilic compounds such as some β-lactams, aminoglycosides, tetracycline and chloramphenicol penetrate the cells [6,8]. Therefore its absence confers high level of resistance against these antibiotics. The *S. marcescens* Omp1 protein was effectively expressed in *E. coli* UH302, a well known porin-deficient strain, as can be seen in the SDS-PAGE gel shown in Fig. 1. This clone (*E. coli* UH302 pOM100) was used (i) to determine the functionality of the protein; and (ii) to purify Omp1 for the biophysical experiments.

Whereas the lack of porins in *E. coli* UH302 results in an extremely low growth rate, the clone grew up much more rapidly (data not shown). As in other papers [17] accumulation experiments of ciprofloxacin into bacteria were used to determine the role of Omp1 in the uptake process. Conclusively these experiments demonstrate that, ciprofloxacin uses Omp1 to penetrate *Serratia* outer surface (Fig. 2)[1]. As can be seen, the expression of Omp1 porin in *E. coli* UH302 leads to an increase in ciprofloxacin accumulation when compared with the parental strain.

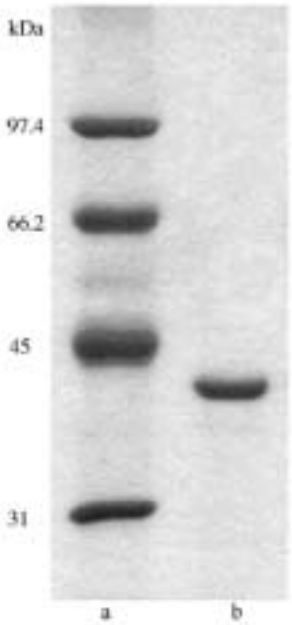

Fig. 4. SDS-PAGE of the Omp1 purification protein from *E. coli* UH302 pOM100. Standard molecular weight (a), Omp1 purified and heated previously (b).

The outer membranes isolated form *E. coli* UH302 and *E. coli* UH302 pOM100 were resuspended in buffer and imaged by AFM (Fig. 3A and B, respectively). The images were taken in tapping mode. Both membranes shown a similar height of 3.72±0.06 nm (n=10) and 3.68±0.12 nm (n=10), respectively, as measured at the edge of the proteolipid sheets. This is consistent with the values reported for other proteolipid sheets [18–20]. While the *E. coli* UH302 outer membranes were flat (Fig. 3A) some protrusions appear at the top of the *E. coli* UH302 pOM100 outer membrane extracts (Fig. 3B). Those protrusions (see Zoom of Fig. 3B) exceed 1.0±0.2 nm (n=20) from the background layer and show an average diameter of 18±3 nm (n=20). This size is higher than the one predicted from our theoretical model [9] and also higher than the values reported for crystals of other porins (i.e. [18,21–23]). However, except

[1] Ciprofloxacin is a fluoroquinolone that can use three different pathways to penetrate the bacteria [7]: (i) an hydrophobic way throughout the lipid bilayer; (ii) a self-promoted still not well known; and (iii) a hydrophilic way due to the channel forming activity of porins. If one of these pathways of entry was not functional (*E. coli* UH302) the ciprofloxacin permeation should be slower.

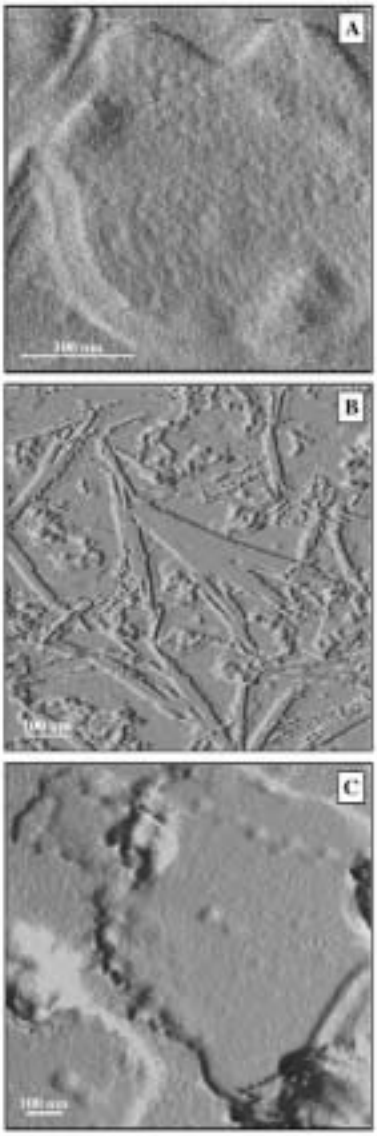

Fig. 5. Atomic force image of amplitude (tapping mode) of Omp1 reconstituted in different lipid matrix. (A) LPS. (B) DMPC. (C) DMPC:POPC (1:1) (mol/mol). Imaging buffer: 20 mM Hepes pH 6.4, 100 mM KCl.

for the purple membrane [24], the cardiac gap junction channels [25] and few more examples in situ crystallization in native membranes is uncommon. On the other hand the protrusions in Fig. 3B could easily result from the self-aggregation of the porin, probably as a trimer. Besides, there is a typical overestimation of the size (about 10%) due to the convolution of the tip [26]. Then introducing this correction [27] the diameter of the protrusions become 5.5±0.9 nm, much more in agreement with the values expected for a porin trimer.

In the reconstitution experiments the purity of the membrane protein is of crucial importance. Therefore we show in Fig. 4 an example of a SDS-PAGE gel of the purified Omp1 protein that has been used in the following experiments. Other variables as pH, ionic strength and several phospholipidic matrices were screened to obtain the best conditions for reconstitution in a biomimetic environment.

In Fig. 5 we show different kind of structures of some of the PLS obtained with different lipid matrices. In Fig. 5A a double layered PLS in a matrix of lipopolysaccharide of *E. coli* is shown. These proteolipid sheets showed a rough surface due to the incorporation of the porin into the layer. However no ordered pattern was observed. On the other hand this matrix it was not possible to flat this PLS on the mica. This roughness becomes a serious difficulty during the scanning of the surface and not allows the correct visualization of a pattern under those experimental conditions.

Two pure phospholipids, POPC and DMPC, with transition temperatures of −2.5 and 24 °C, respectively, were used because they cover the range of temperatures reached during the reconstitution process (see Methods). The Fig. 5B shows the proteolipid sheets formed with pure DMPC as the matrix. The structures observed were tubular-like, showing several states of aggregation. These structures have been observed in other reconstitution experiments [28] and result apparently from the formation of tubular micelles or tubular 2D crystals. Consequently, DMPC was also discarded as lipid matrix for Omp1.

In Fig. 5C an equimolar mixture of DMPC and POPC was used as a matrix. Transition temperature of this mixture ~13 °C was calculated by fluorescence polarization (result not shown). Interestingly, after the incorporation of the porin

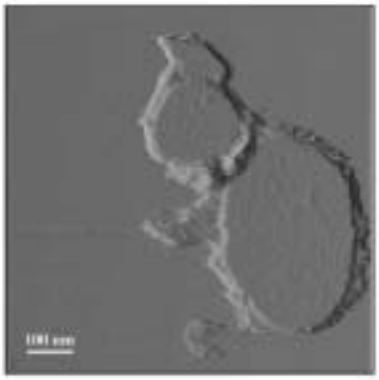

Fig. 6. Atomic force image of deflection (contact mode) of Omp1 reconstituted in POPC at LPR of 0.5. Imaging buffer: 20 mM Hepes pH 6.4, 100 mM KCl.

into the bilayer its fluidity remains constant. Proteolipid sheets formed with DMPC:POPC (Fig. 5C) showed the structure of typical bilayer obtained after spreading. Although no conclusive, certain structures on the top of the bilayer surface, similar to those observed in the natural extract of *E. coli* (Fig. 3B), were observed.

Judging only the quality of the images, the best proteolipid sheets were obtained using pure POPC (Fig. 6). These PLSs are similar to others reported in the literature and obtained with other proteins [18,19]. The PLSs were 10.0 ± 0.3 nm ($n=10$) height, as measured at the edges and can be interpreted as a double layered structure. The deflection image is presented here to enhance this fact. Remarkably a kind of organization is observed at the upper layer which did not reveal a pattern. These PLSs formed are of sufficient rigidity to ensure the contact scan at high resolution without perturbation of the sheet with the tip.

Therefore, our AFM results suggest that POPC was the most suitable lipid matrix to reconstitute the OmpI. These PLSs need to be further investigate, particularly, the origin of the corrugations on its top. At this point we are not able to confirm if these structures are due to the arrangement of the protein in lattices or are originated from self aggregation of the protein. Nowadays, we are studying the optimal conditions to minimize the repulsion over the sheet in order to reach higher resolution images.

Acknowledgements

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Short communication

Atomic force microscopy study of *Escherichia coli* lactose permease proteolipid sheets

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Abstract

Proteolipid sheets (PLSs) obtained using the vesicle fusion technique on a convenient surface are the base to obtain transmembrane protein biosensors. In this preliminary work, we have screened several physicochemical conditions to optimize the visualization of proteolipid sheets formed between different phospholipid matrices and the membrane protein lactose permease (LacP) by atomic force microscopy (AFM).

When LacP was reconstituted in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes, the proteolipid sheets were densely packed with an upper layer that protruded from a background layer. Several lipid protein molar ratios (LPR) were screened. High resolution analysis of the upper layer revealed a quasi-crystalline arrangement formed by small entities that could be attributed to the protein. The approach described here may be suitable for the rational design of biosensors based in other transmembrane proteins.

Keywords: Lactose permease; Phospholipid; Solid-supported proteolipid sheets; Atomic force microscopy

1. Introduction

There is currently considerable interest in using membrane proteins for biosensor applications. The biosensor would consist of a lipid layer (actually a bilayer), in which the membrane protein is embedded as a specific detector that coats a sensor surface. The basic strategy consists of reconstitution of the protein in liposomes to produce proteoliposomes that are subsequently spread onto a convenient surface. This lead to obtain solid-supported proteolipid sheets (PLSs)[1] (Ruiz et al., 2004). In biosensor design (Göpel and Heiduschka, 1995) and also in two-dimensional (2D) crystallization (Kühlbrandt, 1992) of membrane proteins, the goal is to obtain the protein embedded in the lipid bilayer. Whilst in 2D crystallization, the generation of repetitive arrays for structural analysis is required, for biosensor applications this may not be necessary if the protein remains immobilized and maintains its biological activity (i.e. see Fisher and Tjärnhage, 2000).

In earlier studies (Kiefer et al., 1991), it was proposed a biosensor based in the incorporation of membrane transport proteins in a planar bilayer which covers the transducer. Afterwards (Klee et al., 1992), it was demonstrated that the lactose biosensor could be produced based on the transmembrane protein lactose permease (LacP) of *Escherichia coli*. The recent generation of 3D crystals of a mutant of LacP (*LacY/C154G*) (Abramson et al., 2003) has provided conclusive evidence about the molecular mechanism of the protein (for a review, see Kaback et al., 2001). This allows now to understand better the mechanism of action of the lactose-sensor in which characterization and scaling-up difficulties has been recognized (Göpel and Heiduschka, 1995). Alongside this, the 2D crystallization of LacP has been with a fusion protein containing cytochrome b_{562} (*LacY/L6cytb*$_{562}$*/417H6*) specifically engineered for this purpose achieved (Zhuang et al., 1999). Importantly, the conditions used in 2D crystallization could be useful, and may be applicable to the preparation

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[1] We use PLSs in the absence of a consensus definition. Other terminology, like solid-supported bilayers, supported phospholipid bilayers or surface planar bilayers can be found in the literature.

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and characterization of PLSs and biosensors based in other transmembrane proteins. In principle, these were the structures on which the earlier LacP biosensor was based (Klee et al., 1992).

The propensity of the LacP protein to aggregate (Engel et al., 2002) along with its extremely high flexibility raises many questions about the nature, stability and reproducibility of the structures obtained after the deposition of LacP proteoliposomes onto a surface. Before any attempt is made to scale-up such a biosensor, basic information about the structural characteristics of the PLSs will be necessary. Having into account that this biosensor is based on the interaction of lactose with LacP properly folded and embedded within the lipid bilayer, the investigation on the nanostructure of the PLSs appears necessary. Therefore, in the present study we have used atomic force microscopy to visualize the nanostructure of such PLSs after deposition on mica, a common support for imaging biological samples in order to provide information for the rational design of a lactose biosensor.

2. Materials and methods

2.1. Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and *N*-dodecyl-β-D-maltoside (DDM) were purchased from Sigma (St. Louis, MO, USA). All other common chemicals were ACS grade.

2.2. Biological

E.coli T-184 cells [*lacI*$^+O^+Z^-(A)$, *rpsL*, *met*, *thr*, *recA*, *hsdN*, *hsdR/F*, *lacI*$^qO^+Z^{D118}(Y^+A^+)$] were generously provided by Dr. H. Ronald Kaback from the HHMI-UCLA.

2.3. Vesicle preparation and protein reconstitution

Chloroform/methanol (50:50 (v/v)) solutions containing appropriate amounts of lipid were dried under a stream of oxygen-free N_2 in a conical tube and the thin film obtained kept under high vacuum for approximately 3 h to remove organic solvent traces. The suspensions obtained after redispersion of the film in resuspension buffer were sonicated for 20 min and solubilized in DDM, 0.02% (w/v). Afterwards, to extract the detergent, the same protocol used for LacP reconstitution (see below) was applied.

LacP was extracted and purified from the overproducing strain *E.coli* T-184, according to previously described methods (Zhao et al., 1999) and reconstituted using methods described elsewhere (LeCoutre et al., 1997). Modifications to adapt the methods to LacP were introduced; essentially, purified LacP solubilized in DDM was mixed with phospholipids, also in DDM, to obtain an appropriate lipid-to-protein ratio (w/w) and a final lipid concentration of 100 μM. Several steps were then applied to extract the detergent: (i) incubation of the mixture at 4 °C for 1 h; (ii) addition of 100 mg of Bio-Beads and incubation at 4 °C for 4 h; (iii) another addition of 100 mg of Bio-Beads and incubation for 12 h at room temperature; (iv) centrifugation at 12,000 rpm for 1 h; (v) resuspension of the pellet in resuspension buffer (10 mM Tris, pH 7.40; 150 mM NaCl; 20 mM $CaCl_2$; $I = 0.15$ m) and (vi) controlled and linear increase of the temperature up to 37 °C.

2.4. AFM observations

Images were recorded in tapping and contact mode with a commercial Digital Instruments (Santa Barbara, CA) Nanoscope III AFM fitted with a 15 μm scanner (d-scanner). Standard Si_3N_4 tips, with a nominal force constant of 0.1 N/m (Digital Instruments), were used and the forces exerted by the tip were minimized by previously recording force plots for each sample. Images were obtained in situ using an AFM fitted with either a tapping-mode liquid cell or a contact-mode cell. Before every sample, the AFM liquid cell was washed with ethanol and ultra pure water (Milli-Q® reverse osmosis system), and allowed to dry in a N_2 stream. Mica discs (green muscovite mica) were cleaved with scotch and glued onto a Teflon disc using a water-insoluble epoxy. These Teflon discs were glued onto a steel disc and then mounted onto the piezoelectric scanner.

2.5. Preparation of PLSs

The spread of the planar membranes on mica were obtained using the vesicle fusion technique (Merino et al., 2003; Ruiz et al., 2004). Aliquots (50 μL) of reconstituted LacP in proteoliposome samples were pipetted onto freshly cleaved mica, allowed to stabilize at 25 °C (above the transition temperature of the phospholipid mixture) for 30 min (approximately) and then rinsed with divalent cation-free buffer (imaging buffer). The tip was immediately immersed in the buffer. To accomplish all these experiments, it was necessary to drift equilibrate and thermally stabilize the cantilever for 30 min before imaging.

3. Results and discussion

In this work, several physicochemical variables have been screened: the purity of the protein, detergent used for the extraction, ionic strength ($I = 0.05$–0.3 m), presence of monovalent (K^+, Na^+) and bivalent ions (Mg^{+2}, Ca^{+2}) in the resuspension buffer, pH (6.0–8.0) and several lipid matrices. The best images were obtained by following the experimental conditions described in the figure legends, which basically coincide, with those established in the work that reported the unique 2D crystallization of LacP (Zhuang et al., 1999). As discussed elsewhere, the presence of the divalent cation is required for transformation (adsorption, fusion and rupture) of vesicles into surface planar bilayers

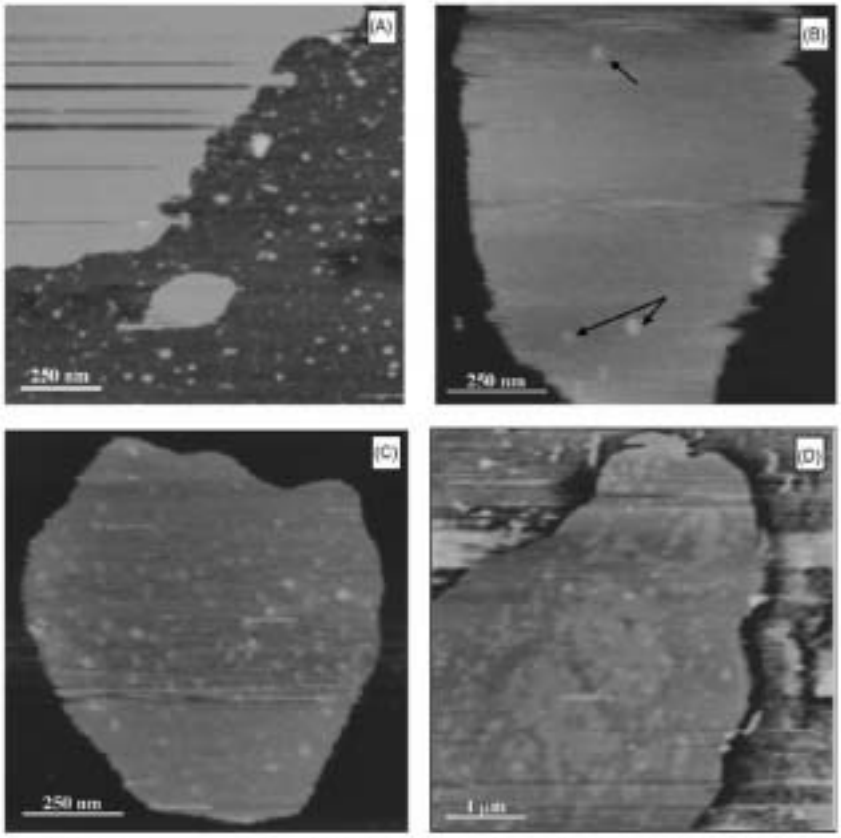

Fig. 1. Images obtained using POPC as lipid matrix and LacP at different values of LPR (w/w): 0 (A), 1.5 (B), 1.0 (C) and 0.5 (D). Images were obtained in tapping mode using as imaging buffer 10 mM Tris–HCl, 150 mM NaCl (pH 7.40) on a mica support. Protrusions in (B) are indicated by black arrows.

(Merino et al., 2003). It is worth noting that one of the crucial points in the transmembrane protein reconstitution arises during the extraction of the detergent (DDM) (for reviews see Rigaud et al., 2000).

Among the phospholipids screened in the present study, POPC, electrically neutral and in fluid phase at room temperature provides an appropriate environment for the correct insertion of the protein (see review of Lee, 2003).

When studying pure POPC bilayers by AFM, we obtained images such as the one shown in Fig. 1. We observed that the lipid layer adsorbed onto mica becomes dramatically affected under the action of the tip (Fig. 1A). Although, the load force of the tip over the sample was adjusted to the minimum value to engage,[2] we observed that the tip scratched the POPC bilayer. However, the height profile analysis (Merino et al., 2003) allows to establish a value of 4.52 ± 0.19 nm for the POPC bilayer which is the height relative to mica. Interestingly, this behaviour is changed by the presence of LacP. As can be seen in Fig. 1B and C, the PLSs remain stable and firmly attached to the substrate under the action of the same load force.

In agreement with the work of Zhuang et al. (1999), crystalline patches with the LacP incorporated were obtained at LPR = 0.5 (Fig. 1D). In the centre, a densely packed layer protrudes from the background layer with some similarities to those depicted in Fig. 1B and C. In this case, however, the upper layer should be attributed to the self-aggregation of the protein (Engel et al., 2002) as a consequence of the gradual decrease of the LPR. A magnified view of the upper layer regions shown in Fig. 1D reveals a quasi-crystalline arrangement (Fig. 2) formed by small entities with a roughness of 0.043 nm.

Although the biological activity of LacP in PLSs has been demonstrated (Ottenhachen et al., 1993), no information about the nature of the adsorbed structures has been, to our knowledge, released to date, (hence, the relevance of Fig. 2).

[2] During scanning, the imaging force is normally adjusted to the smallest possible values, whilst the image remains clear (usually less than 200 pN).

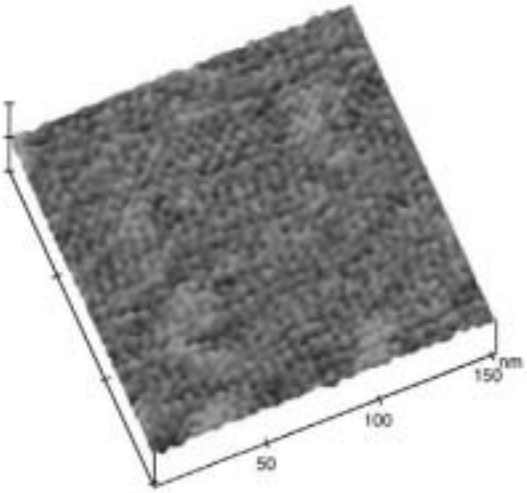

Fig. 2. 3D perspective of an upper layer of a proteolipid LacP sheet formed with POPC at a LPR (w/w) of 0.5 visualized in contact mode using as imaging buffer 10 mM Tris–HCl, 150 mM NaCl (pH 7.40) on a mica support.

The structural aspect has attracted our attention because AFM have opened up the possibility of a direct manipulation (Fotiadis et al., 2002), and eventually nanofabrication of these kind of biosensors based in transmembrane proteins. As an example of the intrinsic difficulties of working with this particular protein, it is worth noting that the poor reproducibility of obtaining 2D crystals of LacP has been recognized (see Fig. 2 in Stahlberg et al., 2001) and that the generation of 3D crystals has resisted more than 20 years of research (Kaback and Wu, 1999). Importantly, an understanding of to know-how to obtain PLSs could have an impact on the design of biosensors based on transmembrane proteins involved, for instance, in antibiotic or antineoplasic resistance and other human diseases associated with membrane transport (Van Bambeke et al., 2000).

The AFM images presented here provide novel insights into the structure of PLSs. Work on the biological activity of the protein adsorbed in several surfaces and other phospholipid matrices is now in progress.

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Effects of Lactose Permease on the Phospholipid Environment in Which It Is Reconstituted: A Fluorescence and Atomic Force Microscopy Study

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The membrane transport protein lactose permease (LacY), a member of the major facilitator superfamily containing 12 membrane-spanning segments connected by hydrophilic loops, was reconstituted in liposomes whose composition was 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol in a 3:1 molar ratio. The structural order of the lipid membranes, in the presence and absence of LacY, was assessed using steady-state fluorescence anisotropy. The features of the anisotropy curves obtained with 1,6-phenyl-1,3,5-hexatriene and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate suggest a surface effect of LacY on the membranes. Atomic force microscopy imaging of supported planar bilayers (SPBs) deposited onto mica was used to examine the effect of LacY on the nanostructure of the phospholipid matrix. Two separated domains were observed in SPBs formed from pure phospholipid mixture. Protein assemblies segregated from the rest of the matrix were observed after the extension of proteoliposomes. The effect of the protein on the electrostatic surface potential of the bilayer was also examined using a fluorescent pH indicator, 4-heptadecyl-7-hydroxycoumarin. Changes in surface potential were enhanced in the presence of the substrate (i.e., lactose). Taken together the results indicate that LacY is segregated into the phospholipid matrix and has moderate effects on the acyl chain order of the bilayers. The changes in surface electrical properties of the bilayers suggest a role for the phospholipid headgroups in proton transfer to the amino acids involved in substrate translocation.

Introduction

Membrane proteins account for over 25% of total cell proteins.[1] 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 transport functions. Among them, lactose permease (LacY),[2] one of the best 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 what is termed the major facilitator superfamily (MFS),[3] most of whose members are predicted to contain 12 transmembrane segments. The secondary structure of LacY consists of 12 transmembrane α-helices, crossing the membrane in a zigzag fashion, that are connected by 11 relatively hydrophilic, periplasmic, and cytoplasmic loops, with both amino and carboxyl termini on the cytoplasmic surface (Figure 1). A three-dimensional (3D) model of a LacY mutant (Cys 154→Gly) and a reaction mechanism derived from X-ray diffraction studies is now available,[4] culminating years of effort. This mechanism confirms the basic structural features elucidated by noncrystallographic approaches[5] based on a combination of molecular biology and various types of spectroscopy.

The physiological activity of the transmembrane proteins may be influenced by or dependent on the physical properties of neighboring phospholipids.[6] Such dependence has been demonstrated in the case of, among others, β-hydroxybutyrate dehydrogenase, an enzyme integrated into the mitochondrial inner membrane, Ca^{2+}−ATPase,[7] melibiose permease[8] from *E. coli*, another member of the MFS, and LacY itself,[9] for which the requirement of phosphatidylethanolamine in functions in vivo has been clearly demonstrated.[10] This phospholipid is also required for the correct folding of the protein.[11] Hence, an understanding of the physicochemical properties and the influence of the phospholipids that constitute the matrix in which the transmembrane proteins are embedded is of critical importance.

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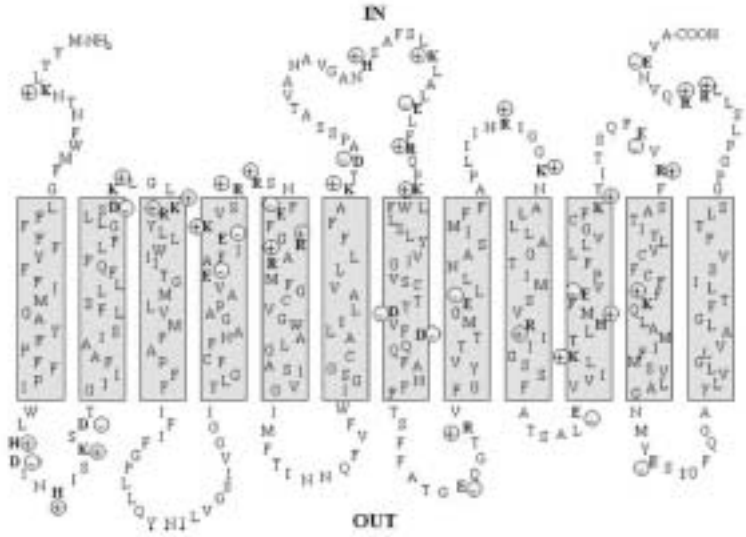

Figure 1. Secondary structure of lactose permease from *Escherichia coli* showing the charged residues (boldface). The one-letter amino acid code is used. The putative transmembrane helices are shown in boxes that are connected by hydrophilic loops.

Here we examine the effects of LacY on the structural order of lipid membranes by measuring, as a function of temperature, the steady-state fluorescence anisotropy (r_s) of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) and 1,6-phenyl-1,3,5-hexatriene (DPH) incorporated into liposomes and proteoliposomes of LacY with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) as the phospholipid matrix.

Recent studies[12] have shown that the insertion of LacY into proteoliposomes depends on the presence of phospholipids in the fluid phase at the temperature of reconstitution. Therefore, to both visualize and elucidate lipid phase behavior, atomic force microscopy (AFM) was used to study the supported planar bilayers formed from POPE/POPG liposomes and proteoliposomes of LacY.

Although the protein becomes randomly oriented when it is reconstituted in liposomes, its presence might affect the electrostatic surface potential of the membranes. The protein contribution to the membrane electrostatic surface potential can be assessed by using a fluorescent pH indicator, 4-heptadecyl-7-hydroxycoumarin (HHC),[13] which has been extensively used in determining the electrostatic surface potential of monolayers[14] and liposomes.[15] Its fluorometric titration, when incorporated in liposomes, facilitates determination of the interfacial pK of the probe and calculation of variation in the electrostatic surface potential ($\Delta\psi$)[4,6] after reconstitution of LacY in the same phospholipid matrix. This information helps us to relate the electrical properties of the phospholipid bilayer surface and LacY activity.

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Materials and Methods

Chemicals. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) 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*-toluenesulfonate (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). 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). All other common chemicals were ACS grade.

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 from the HHMI−UCLA. LacY was extracted and purified from the overproducing strain *E. coli* T-184, according to previous studies.[17] 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 0.5 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 was purified by affinity chromatography on immobilized monomeric avidin beads, as described previously.[18] The column was washed with approximately 200 mL of 50 mM NaP_i, 150 mM NaCl, pH 7.50, 0.02% DDM, and the permease was 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).

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-

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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 KPi buffer, pH 7.50, and sonication for 30 min in a bath sonicator. The liposomes were then dissolved in 0.5% (w/v) of DDM and mixed with the solubilized protein 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).[19] The first two extractions were performed at room temperature for 2 h each, and the last extraction was performed at 4 °C overnight. The vesicles were collected by centrifugation for 4 h at 35000 rpm and resuspended in a small volume of 50 mM KPi 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 a brief period of sonification in a bath sonicator at a temperature below 37 °C.

Steady-State Anisotropy Experiments. The experimental procedures to measure the steady-state anisotropy of TMA-DPH and DPH were adapted from previous studies.[3,12,21] 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 three degree intervals in the range between 0 and 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 unlabeled suspension. Steady-state anisotropy (r_s) values were calculated according to

$$r_s = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \qquad \text{(I)}$$

where I_{VV} and I_{VH} 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 following equation was fitted to the anisotropy versus temperature data

$$r_s = r_{s2} + \frac{r_{s1} - r_{s2}}{1 + 10^{B'(1/T - 1/T_m)}} \qquad \text{(II)}$$

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 (E) by $E = [1 - 1/(1 + B')]$; the introduction of B yields a convenient scale of cooperativity ranging from 0 to 1.

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.[18] Briefly, in these experiments the molar ratio of total lipid to fluorescent probe was 1000:1. Similar molar ratios and ionic strength were maintained in the experiments carried out in the presence of LacY in order to calculate $\Delta p\bar{K}$ 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

$$\Delta\psi = \frac{-2.3RT}{F}(\Delta p\bar{K} - \Delta p\bar{K}_0) \qquad \text{(III)}$$

where $\Delta p\bar{K}_0$ is the difference between the $p\bar{K}$ determined in a electrically neutral liposome—that we take as the reference (POPC)—and the $p\bar{K}$ of the probe in solution and $\Delta p\bar{K}$ is the difference between the $p\bar{K}$ determined in POPE:POPG (3:1, mol/mol), of either the liposome or proteoliposome system, and the $p\bar{K}$ of the probe in solution. R is the universal gas constant, T is the temperature (310 K), and F is Faraday's constant.

Supported Planar Bilayer Formation and Atomic Force Microscopy Observations. The spread of the supported planar bilayers was obtained by using the vesicle fusion technique described elsewhere.[12] Briefly, 80 μL of either liposomes or proteoliposomes in 10 mM Hepes pH 7.40, 150 mM NaCl, 20 mM $CaCl_2$; $I = 0.19$ n buffer, was pipetted onto freshly cleaved mica and incubated for 10 min at room temperature, before being washed with 10 mM Hepes pH 7.40, 150 mM NaCl; $I = 0.15$ n (image buffer). The tip was immediately immersed into the liquid cell. To perform all these experiments it was necessary to drift equilibrate and thermally stabilize the cantilever for 30 min in the presence of buffer.

All images were recorded in tapping mode with a commercial Digital Instruments (Santa Barbara, CA) Nanoscope III AFM fitted with a 15 μm scanner (d-scanner). Standard Si_3N_4 tips, with a nominal force constant of 0.1 N/m (Digital Instruments), were used, and the forces were minimized during the scans. Before every sample, the AFM liquid cell was washed with ethanol and ultrapure water (Milli Q reverse osmosis system) and allowed to dry in a N_2 stream. Mica (green muscovite mica) was cleaved with Scotch tape and glued onto a Teflon disk by a water-insoluble epoxy. These Teflon disks were glued onto a steel disk and then mounted on to the piezoelectric scanner. All images were scanned under aqueous solution. Prior to imaging the sample, the tip was stabilized in buffer by scanning of the mica. Force plots were recorded for every sample to control the repulsion of the tip, and images were flattened with the Nanoscope III program.

Results

The temperature dependence of TMA-DPH and DPH liposomes and proteoliposomes is shown in Figure 2. The features of the phospholipid phase transition, for TMA-DPH (Figure 2A) and DPH (Figure 2B) liposomes, were almost unaffected by the presence of LacY at a protein-to-lipid molar ratio of 1:3000. However, the anisotropy of DPH dropped to lower values than those observed with TMA-DPH for the same temperature increases. Thus, the anisotropy of TMA-DPH dropped from 0.34 to 0.24, approximately, when the temperature increased from 0 to 35 °C, while that of DPH dropped from 0.32 to 0.13 for the same temperature range.

Parameters T_m and B for the TMA-DPH liposomes, calculated according to described methods, are listed in Table 1. As can be seen, LacY caused an approximate 0.3 °C increase in the temperature of the phospholipid phase transition (T_m) and a small change in B. Conversely, the presence of LacY induces a decrease of 1.6 °C in the T_m and a lowering of B when DPH was the probe used.

The topography of the supported planar bilayer (SPB) of the POPE/POPG phospholipid matrix is shown in Figure 3A and reveals the existence of two different domains (see differences in contrast). Line profile analysis, such as the one shown in Figure 3B, allows us to establish that (i) the height of the bilayer is 4.4 ± 0.2 nm ($n = 10$), by measuring it at the edge of the SPB and (ii) there is a difference of 0.64 ± 0.07 nm ($n = 10$) between the upper and the lower domain (see Figure 3B). Figure 3C shows the AFM topographic image of a proteolipid sheet of POPE/POPG and LacY at a protein-to-lipid molar ratio of 1:30. Here, two regions or domains are observed underlying supporting mica (white asterisk in Figure 3C). The upper domain regions (white star) constitute the largest region while the lower domain forms small patches (black asterisk). This can be corroborated by the line profile analysis shown in Figure 3D, which corresponds to the line drawn in Figure 3C. When a part of this image is magnified

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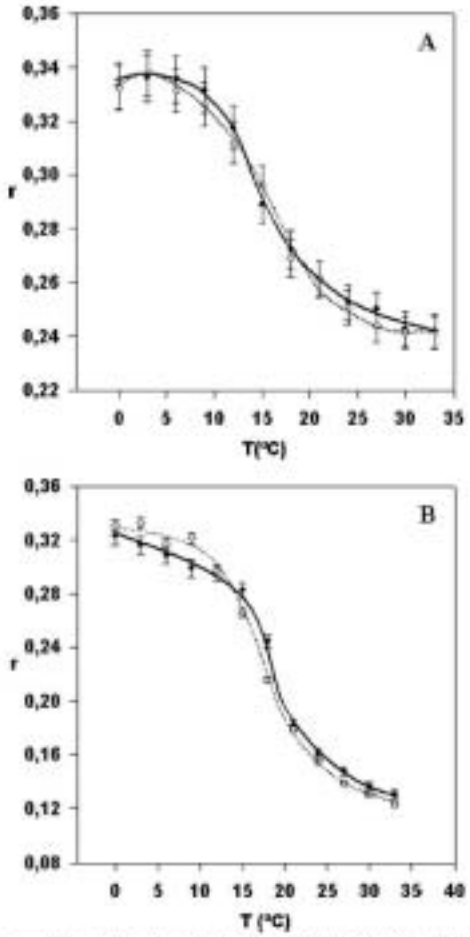

Figure 2. Steady-state polarization of TMA-DPH (A) and DPH (B) for vesicles formed with POPE/POPG (3:1, mol/mol). Liposomes (filled circles) and proteoliposomes of LacY (open diamonds) are shown. Each point indicates the mean value of three experiments. Data were fitted to sigmoid curves as described in the methods section.

Table 1. Transition Temperature (T_m) and Cooperativity (B) Values Obtained by the Nonlinear Adjustment of Steady-State Polarization Data from Figure 2[a]

	liposomes		proteoliposomes of LacY	
probe	T_m (°C)	B	T_m (°C)	B
TMA-DPH	15.4 ± 0.4	0.69	15.7 ± 0.4	0.67
DPH	18.9 ± 0.4	0.74	17.3 ± 0.2	0.68

[a] Results represent data of three independent experiments.

(Figure 4), we can observe how the upper domain is covered by round protrusions, segregated from the lower domain, in a quasi-crystalline arrangement. It can be seen that the lower domain region shows a featureless surface.

The surface pK values and electrical surface potentials ($\Delta\psi$) calculated according to eq III are shown in Table 2. The reference surface potential (0 mV) was assigned to the neutral phospholipid (POPC). As expected, the introduction of the negatively charged phospholipid POPG into the bilayer leads to a negative value of $\Delta\psi$ being obtained. As can be seen in Table 2 the values obtained for the proteoliposomes were positive. It should be noted that when lactose was added (10 mM) the surface potential shifted to even more positive values, +103.35 mV. Similar changes were obtained by addition of the analogue substrate TDG. The addition of sucrose did not significantly affect the surface potential values.

Discussion

The experiments presented in this paper were designed to investigate the effects of LacY insertion into lipid bilayers (liposomes and SPBs) where it is reconstituted. It has been demonstrated that LacY is fully functional when reconstituted in native *E. coli* extract membranes[22,23] and binary mixtures of phosphatidylglycerol and phosphatidylethanolamine.[9,24] For this reason we selected POPE/POPG (3:1, mol/mol) as a suitable phospholipid matrix.

We measured the fluorescence anisotropy of TMA-DPH and DPH in liposomes and proteoliposomes. The changes in the features of the phospholipid phase transition in the presence of LacY were far from dramatic. To explore the effects of LacY on distinct regions of the bilayer, we employ the fluorescent dyes TMA-DPH and DPH. TMA-DPH remains anchored at the aqueous interface of the phospholipids bilayer and as can be seen there was little to no effect of LacY on TMA-DPH anisotropy. On the other hand, significant changes with the more deeply embedded DPH dye were observed. It is important to mention here that for these experiments transmembrane proteins are normally reconstituted into proteoliposomes at low protein-to-lipid ratio.[20] Thus, most of DPH and TMA-DPH molecules would be in lipid a long way from the protein in the membrane. Hence, the measurable and repetitive changes in T_m and B that we have detected would become significant if higher protein concentrations, as those used to form SPBs, could be used in this method. Actually, if we assume that T_m might respond linearly with increasing concentrations of the protein,[25] the decrease of T_m calculated from DPH anisotropy measurements would reflect that there is a moderate change in the phospholipid acyl chain order in the presence of LacY. In addition, the slight increase of T_m in TMA-DPH liposomes in the presence of LacY suggest that hydrogen bonds might be formed between the headgroups of the phospholipids and some residues in the putative loops, most of them positive[26] at physiological pH (see Figure 1). A note of caution, however, should be added with regard to this interpretation.

The characteristics of the SPBs formed from liposomes should be different from those formed from proteoliposomes. Despite the fact that separate domains cannot be observed in POPE/POPG bilayers using solid-state NMR at 30 °C,[27] we unambiguously observed two different domains in the SPBs formed with the phospholipid matrix

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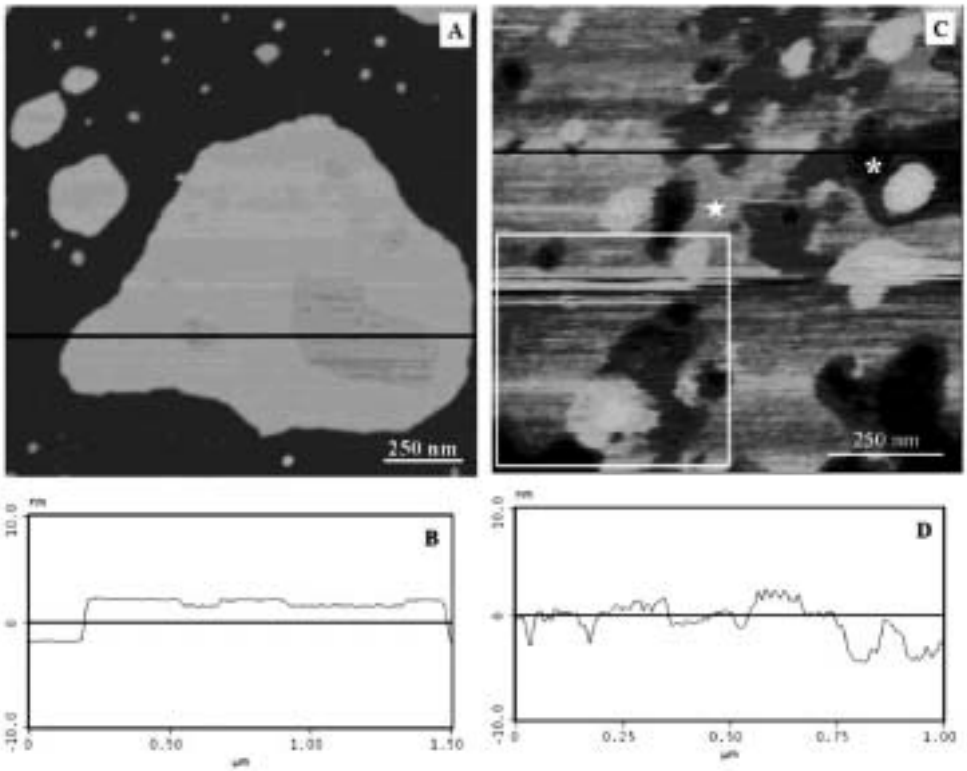

Figure 3. Topography images obtained from the extension of POPE/POPG (3:1, mol/mol) by AFM (A) liposomes and (B) LacY proteliposomes: upper domain (white star), lower domain (black asterisk), bare mica (white asterisk). Images were obtained in tapping mode using imaging buffer. Cross sections measured along the black lines are presented for each image (B) and (D), respectively.

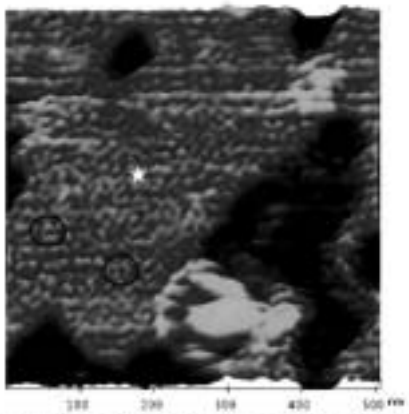

Figure 4. Higher magnification image of the inset in Figure 3.

under study. Two findings allow us to assign the higher level to POPE and the lower level to POPG: (i) the area fraction covered by the lower domain is in agreement with the nominal composition of the liposome preparation and (ii) the POPE molecule is slightly longer than that of POPG due to differences in the size and orientation of the headgroup.[13] Such domains could be interpreted at the molecular level by taking into account, first, that POPE readily forms hydrogen bonds[29] and, second, that POPG may form clusters.[30]

Table 2. Experimental Electrostatic Surface Potentials Calculated Using Equation III[a]

	liposomes		proteliposomes of LacY	
	pK	Δψ (mV)	pK	Δψ (mV)
POPC	8.13 ± 0.07	0		
POPE/POPG (3:1)	8.59 ± 0.19	−28 ± 12	6.92 ± 0.18	+74 ± 12

[a] Data are means of ± standard deviation of three independent experiments.

Importantly, AFM provided evidence of protein assemblies which are unambiguously segregated from the rest of the phospholipid matrix. This observation is in agreement both with other studies[31] that suggest the formation of phospholipid domains in the presence of LacY and with the segregation of LacY in SPBs of POPC.[12] For other proteins and peptides such an observation could be

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the result of the favored insertion into fluid-phase domains.[32,33] However, this cannot be the case here because POPE and POPG are both in the fluid phase at the temperature of reconstitution.

The high hydrophobicity of LacY (12 α-helices embedded in the phospholipid matrix) is likely to be the main force that drives the final integration of the protein within the bilayer. In agreement with such hydrophobicity the protein tends to self-aggregate,[34] resulting in the formation of enriched domains such as those observed here. This would explain why the features of the phospholipid phase change are almost unaffected as can be observed in our anisotropy measurements. However, a marginal but measurable electrostatic interaction indicates that hydrogen bonding might exist between LacY and the phospholipid headgroups. This is reasonable considering both the high hydrophobicity of LacY and the interfacial properties of the phospholipids studied.[35] Moreover, it is quite conceivable that due to the positive charge exposed to the interface by the protein, the lipid ring immediately surrounding the protein should be enriched in the negatively charged phospholipid (POPG). It is interesting to recall that this phospholipid, as well as the POPE,[36] may establish intermolecular hydrogen bonding.[34] Thus, in a bioenergetic context, hydrogen bonding should be related to the so-called "microlocalized" chemiosmotic scheme, which basically assumes the existence of a protonic network at the membrane interface.[37] Thus, our observation that hydrogen bonding may occur at the protein−membrane interface supports the idea that either PE or PG might transfer the proton via a hydrogen bond to the amino acid or amino acids[38] involved in the translocation (probably Glu 269 and His 322).

Finally, from the changes in the electrostatic surface potential observed when lactose and TDG are added to charged proteoliposomes, it can be assumed that LacY undergoes a conformational change. This change fits with the proposed mechanism of action[3] and is assumed to arise from the binding of the substrate to the protein. Although indirectly, this demonstrates that LacY is only active in the presence of POPG and POPE because those changes were not observed with neutral phospholipid matrixes (data not shown).

The effects of LacY on other phospholipid matrixes, neutral and charged, are currently in progress in our laboratory, and the results will be shown in a subsequent paper.

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9.2. CONTRIBUCIONES A CONGRESOS

- Domènech, Ò.; Merino, S.; Hernández-Borrell, J.; Montero, M.T. Contribución oral: *Interaction Protein-Membrane Models: an AFM Study*. III Congreso Español de Microscopía de Fuerzas y Efecto Túnel. Zamora (España), del 24 al 27 de septiembre de 2002.
- Merino, S.; Domènech, Ò.; Viñas, M.; Montero, M.T.; Hernández-Borrell, J. Póster: *Lactose permease affects the phospholipid environment in which it is reconstituted: an atomic force microscopy study. 3rd Portuguese-Spanish Biophysics Congress*. Lisboa (Portugal), del 29 de octubre al 1 de noviembre de 2004.
- Merino, S.; Domènech, Ò.; Montero, M.T.; Hernández-Borrell, J. Póster: *Two-dimensional crystals of lactose permease of Escherichia coli observed by using the atomic force microscope*. *VIth European Symposium of The Protein Society*. Barcelona (España), del 30 de abril al 4 de mayo de 2005.

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