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Preliminary atomic force microscopy study of two-dimensional crystals of lactose permease from *Escherichia coli*

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Abstract

Lactose permease (LacY) of *Escherichia coli* is not only a paradigm for secondary transporters but also for difficulties in two-dimensional (2D) crystallization. In this work we present the progresses achieved in the observation of 2D crystals of wild-type LacY by atomic force microscopy (AFM). Crystals were obtained following reconstitution of LacY in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes. Proteolipid sheets (PLSs) 6.4 nm in height were obtained after spreading the samples onto mica. Observations were carried out in liquid medium and in contact mode (CM-AFM). When the crystalline surfaces of the PLSs were imaged regular packing arrangements were observed. The back-Fourier transformation revealed the existence of various orientations mostly consistent with crystals possessing $p2$ symmetry and unit-cell dimensions: $a = 13.15$ nm, $b = 16.74$ nm, $\gamma = 116°$. The characteristics, size, and shape of the repetitive motif could be compatible with dimers of this protein. These preliminary results are compared and discussed with previously reported 2D crystals observed by electron microscopy.

Keywords: Lactose permease; Proteolipid sheets; Atomic force microscopy; Two-dimensional crystallization

1. Introduction

The lactose permease (LacY) of *Escherichia coli*, which is encoded by the *lac y* gene, the second structural gene in the *lac* operon [1], is an integral membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ across the cytoplasmic membrane. LacY belongs to what is called the major facilitator superfamily (MFS) [2], most of whose members are estimated to contain 12 transmembrane segments. LacY is one of the most studied cytoplasmic membrane proteins and is often used as a paradigm for those secondary transport proteins that couple the energy stored in an electrochemical ion gradient to a concentration gradient.

Interest in membrane transport proteins, including channels, results from the fact that a large number of them are involved in physiological and pathological processes. Furthermore, at least two of the most widely prescribed drugs in the world, fluoxetine (Prozac) and omeprazole (Prilosec), are targets of membrane-transport proteins. No less compelling is their role in drug-resistance antibiotics and anti-cancer agents, where membrane proteins form the basis of the well-known efflux–pump mechanism [3].

The secondary structure of LacY (Fig. 1a) consists of 12 transmembrane α-helices, crossing the membrane in a zigzag fashion, and connected by 12 relatively hydrophilic, periplasmic and cytoplasmic loops, with both amino and carboxyl termini present on the cytoplasmic surface.

Abbreviations: LacY, lactose permease; PLSs, proteolipid sheets; CM-AFM, contact mode-atomic force microscopy; 2D, two-dimensional; 3D, three-dimensional.

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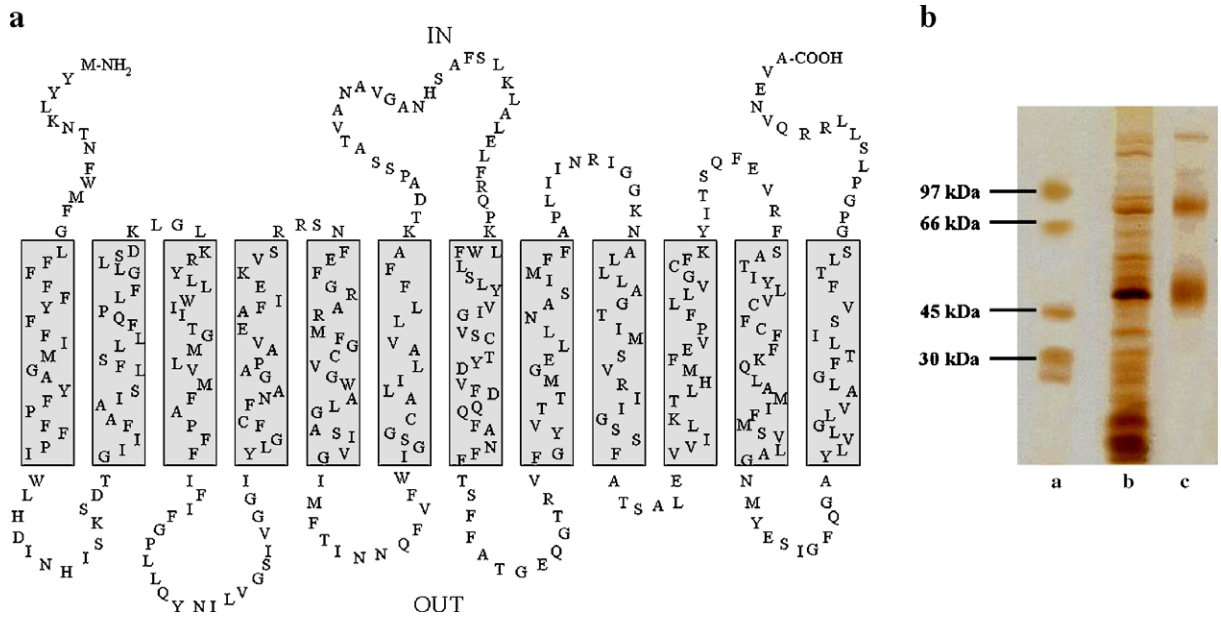

Fig. 1. (a) Secondary structure of lactose permease of *Escherichia coli*. The one-letter amino acid code is used. The transmembrane helices are shown in boxes that are connected by hydrophilic loops. (b) SDS-PAGE (10% running gel and 4% stacking gel, stained with silver nitrate) analysis of samples from various stages of LacP purification procedures. Lane a: molecular weight markers (Mw indicated in kDa); lane b: membrane extract of total membrane proteins; lane c: purified LacY, obtained following Ni-NTA chromatography.

For more than a decade, a battery of techniques combining molecular biology with several biochemical and biophysical studies provided indirect information on the tertiary structure of LacY. These studies served as the basis for an early model of the transport mechanism [4]. However, repeated failures to obtain three-dimensional (3D) crystals, even using fusion proteins containing soluble domains inserted into various loops of LacY [5] and other strategies,[1] remained a basic limitation in determining any precise structural information on the mechanisms underlying protein activity [6].

It was not until very recently that 3D crystals from a mutant of LacY, with Gly in place of Cys154 (Cys154→Gly154; C154G), were obtained of sufficient quality for high resolution X-ray diffraction studies [7]. A crystal structure for C154G was then derived and a plausible mechanism for lactose/proton symport suggested.

In parallel with these classical 3D crystallization studies, attempts to obtain highly ordered two-dimensional (2D) crystals were undertaken with varying outcomes. Thus, while filamentous arrangements were observed in a very earlier study [8], 2D crystals were obtained only after a lengthy series of trials involving different phospholipid matrices and conditions [9]. In both cases, however, observations were carried out under non-biomimetical conditions; that is, through electron microscopy (EM), in a vacuum, and with samples stained with heavy metal solutions. Additionally, the authors recognized that 2D crystallization could be difficult to reproduce [10], attributable to the protein's high hydrophobicity and flexibility. Related with this in previous works we revisited the experimental conditions to reconstitute LacY into proteolipid sheets (PLSs) and found that protein incorporation is highly dependent of the presence of phospholipids with low temperature of transition [11–13].

Herein we report the successful realization of 2D crystals of the wild-type permease into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) phospholipid matrix. The principal achievement of the present work was, however, to image the 2D crystals of LacY in a physiological medium using atomic force microscopy (AFM). The images revealed regular packing arrangements mostly consistent with a *p*2 crystal symmetry, whose dimensions were in close agreement with values obtained by electron microscopy [9]. Projection maps suggest that a dimer of LacY could be the observed motif. This preliminary work provides new perspectives for obtaining high-resolution images in the near future that will allow a better visualization of individual entities and eventually manipulation of LacY under biomimetical conditions by AFM.

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 Chemical Co., St. Louis, MO, USA. All other

[1] Among others, crystallization attempts made in presence of divalent metals with mutants containing 6 contiguous His residues or co-crystallization of LacY with the enzyme IIAglc (R. Kaback, personal communication).

common chemicals were ACS grade. Bio-Beads were purchased from BioRad.

2.2. Bacterial strain

Escherichia 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.

2.3. Vesicle preparation and protein reconstitution

Chloroform/methanol (50:50, v/v) solutions containing the necessary quantity of lipid were dried under a stream of oxygen-free N_2 in a conical tube, and the thin film obtained was kept under high vacuum for approximately 3 h to remove organic solvent traces. The suspensions obtained after re-dispersion of the film in resuspension buffer (RB) (10 mM Tris–HCl pH 7.40, 150 mM NaCl, 40 mM $MgCl_2$) were sonicated for 20 min and solubilized in DDM 0.02% (w/v). LacY was extracted and purified from overproducing the strain *E. coli* T-184 according to accepted procedures [14,15], and then reconstituted according to methods previously described [13]. Briefly, purified LacY solubilized in DDM (see SDS–PAGE gel in Fig. 1) was mixed with phospholipids, also in DDM, to obtain the proper lipid-to-protein ratio (LPR) (w/w) for a lipid final concentration of 100 μM. The following steps were taken 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) a second addition of 100 mg of Bio-Beads and incubation overnight at room temperature; (iv) centrifugation 12,000×*g* for 1 h; (v) resuspension of the pellet in RB; and (vi) controlled and linear increase of the temperature up to 37 °C.

2.4. AFM observations

Images were recorded in contact mode (CM-AFM) 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/m (Digital Instruments), were used and force exertion was minimized by recording beforehand force plots for each sample. Images were obtained in situ using an AFM fitted with a 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 an N_2 stream. Mica discs (green muscovite mica) were cleaved with scotch tape and glued onto a Teflon disc by a water-insoluble epoxy. These Teflon discs were then glued onto a steel disc and finally mounted onto the piezoelectric scanner.

The spread of the planar membranes on mica was obtained using the vesicle fusion technique [16]. As previously described [17], aliquots of liposome or reconstituted LacY in proteoliposomes samples were pipetted onto freshly cleaved mica, allowing samples to stabilize at 25 °C (above the transition temperature of the phospholipid mixture) for 30 min (approx.) before rinsing surfaces with imaging buffer (IB) (10 mM Tris–HCl pH 7.40, 150 mM NaCl). Tips were immediately immersed in buffer. To accomplish these experiments it was necessary to drift-equilibrate and thermally stabilize the cantilever for 30 min before imaging.

2.5. Image processing

Images were flattened using Nanoscope III software. Suitable areas were digitized and cell morphology assessed by Fourier peak filtration.

3. Results and discussion

In Fig. 1b we present a silver-stained gel, which was derived from SDS–PAGE for the total membrane protein

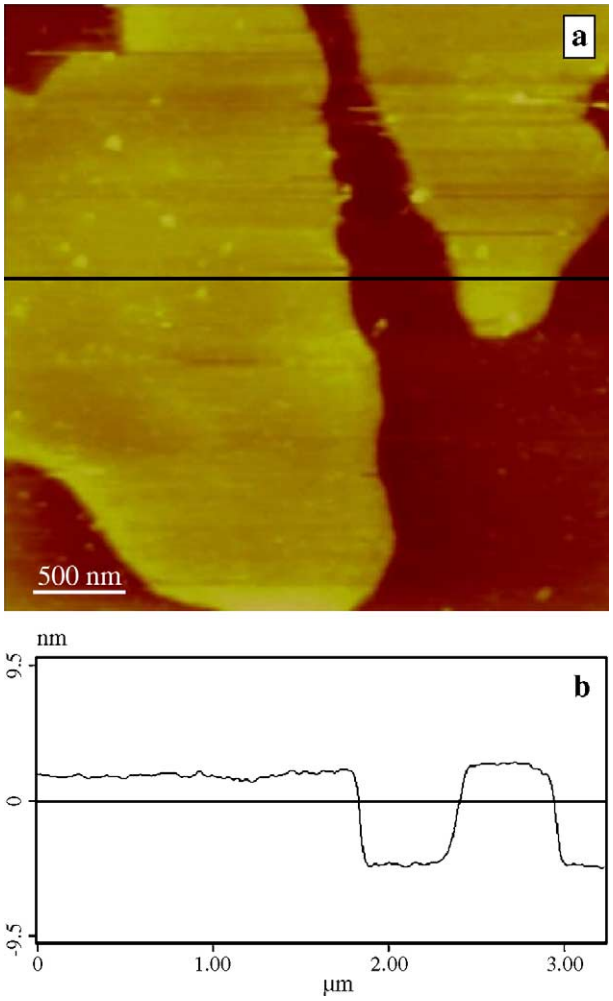

Fig. 2. CM-AFM image of a proteolipid sheet constituted by POPC and LacY at a LPR of 0.5 (w/w). Imaging buffer was 10 mM Tris–HCl pH 7.40; 150 mM NaCl. (a) Image of topography. (b) Cross-section of the topography image.

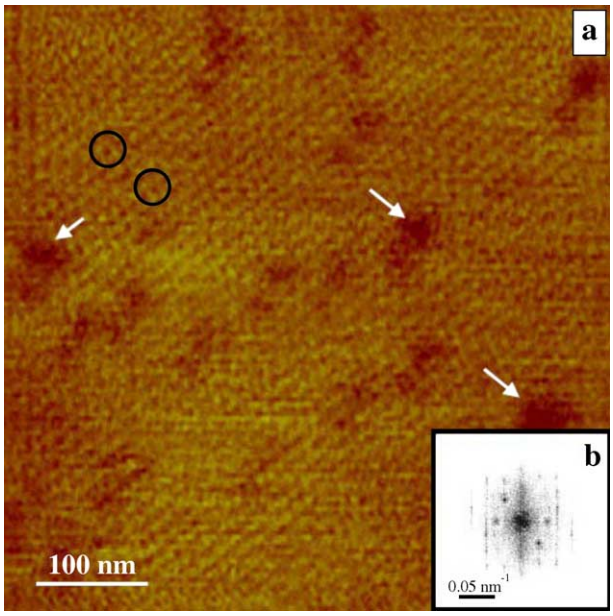

Fig. 3. Surface topography of a proteolipid sheet of POPC and LacP at a LPR of 0.5 visualized in contact mode using 10 mM Tris–HCl pH 7.40; 150 mM NaCl as imaging buffer. (a) Topography image of the 2D crystal. White arrows show some defects of the crystal and black circles show the sub-units of the crystal. (b) Computer-generated Fourier transformation of image (a).

fraction (lane b), and then purified following Ni-NTA (lane c) chromatography. In addition to the major band corresponding to lactose permease monomer (Fig. 1), minor bands migrating at positions of dimer and trimer, respectively, were visible. The apparent molecular mass of the monomer was 47 kDa, consistent with previous studies [14,15]. The presence of dimers, as well as the tendency of LacY to self-aggregate, has been discussed elsewhere [18,19].

At low magnification, room temperature, LPR of 0.5, and in aqueous solution (IB), CM-AFM topography revealed large PLSs such as those shown in Fig. 2a. From the cross-section shown in Fig. 2b it is evident that PLSs are 6.4 ± 0.3 nm (n = 30) high and remarkably flat. This high value is consistent with the thicknesses of 2D crystals for different membrane proteins previously described [20–22]. The few randomly distributed round objects can be attributed to excess lipid adsorbed onto the PLSs. The image quality of biological samples using CM-AFM appears to depend on the high lateral forces derived from the scanning motion [23] as well as the ionic strength of the visualization buffer [13,20]. This might explain the differences in the tonality coloration of Fig. 2a which is, however, similar to other biological samples imaged in liquid medium [11,13,17].

At higher magnification, 2D crystal packing is clearly recognizable (Fig. 3a). The large crystalline surfaces that do not fill the total area of the PLSs were imaged at molecular resolution. Defects (white arrows) were frequently observed. The computer generated Fourier transformation of this area (Fig. 3b, inset) showed peaks extending to 1/6 nm^{-1}. Some spots, however, are weak and others appear offset by a single lattice. A filtered image of such crystal, calculated by back-Fourier transformation, is shown in Fig. 4a. Although it is obvious that the entire area is not a single crystal and assuming that the protein is correctly folded into the POPC bilayer [24] most of these regions appear to possess $p2$ symmetry with unit-cell dimensions: a = 13.15 nm, b = 16.74 nm, γ = 116°. The corresponding 2D projection map (Fig. 4b) indicates that these crystals are made by filamentous arrays similar to lactose permease associations previously reported [8]. On the other hand, the shape of the Wigner–Seitz cell (Fig. 4c) suggests the existence of an asymmetric dimer-like motif. When interpreting this motif one can assume that

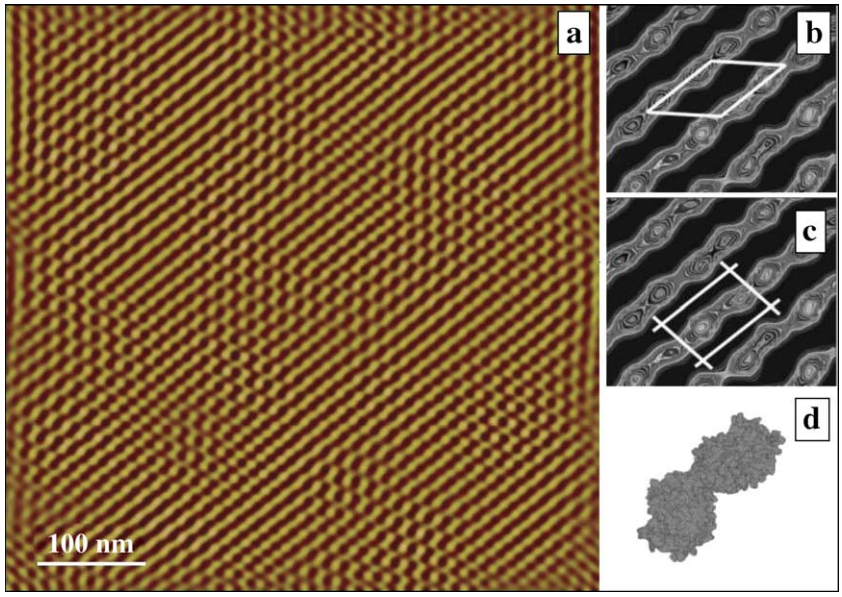

Fig. 4. (a) Back-Fourier transformation of Fig. 2a produced by computer filtering. (b) One unit-cell and the projection map obtained by imposing $p2$ symmetry. (c) Wigner–Seitz cell. (d) View of the surface of a dimer along the membrane normal, as constructed by Protein Explorer.

dimer asymmetry results from a random orientation of LacY when reconstituted in the phospholipid matrix. To illustrate this hypothesis, the view along the membrane normal of a model dimer, constructed with Protein Explorer [25] and using the atomic coordinates from the RCSB protein data bank code (1PV6), is shown in Fig. 4d.

With the objective to promote the capacity of LacY to form crystal lattices a fusion protein consisting of lactose permease with cytochrome b_{562} in the middle cytoplasmic loop and 6 residues at the C terminus (LacY/L6cytb$_{562}$/417H6 known as "red permease") was engineered [5]. As previously reported by Zhuang et al. [9], different packing arrangements of "red permease" crystals can be obtained depending on reconstitution conditions: (i) tetragonal lattices, in optimum order, with unit-cell vectors of $a=9.9$ nm, $b=17.4$ nm; and (ii) trigonal lattices with unit-cell vectors of $a=b=10.3\pm0.6$ nm and $a=b=13.7\pm1$ nm, respectively, and different morphologies. While the values for our unit-cell fell within the same order of magnitude as those obtained by EM, discrepancies arose in the interpretation of projection maps. Thus, as shown in Fig. 4c, by fitting the motif to an ellipse, a surface area of 45 ± 3 nm^2 ($n=30$) was calculated. However, in the projection maps of the EM study by Zhuang et al. [9], the unit-cell, whether trigonal or rectangular, housed trimeric complexes of approximately 11 nm in diameter.

Besides the differences in quality of the Fourier transformation, which are clearly limited by the quality of the 2D crystals obtained as well as by the well known difficulties in reproducibility [13], several other factors might explain the variations in size and shape of the subunits. Thus: (i) "red permease" (69 kDa) was used instead of the wild-type permease (46 kDa); (ii) the sample was negatively stained for EM observation, which was not the case for AFM; and (iii) our observations were carried out using CM-AFM in liquid medium, versus in a vacuum.

Remarkably, our results demonstrate that "red permease" is not required for crystallization. However, until more conclusive data supporting our interpretation is compiled, the only 3D crystal reported to date [7] remains that similarly obtained in dimeric form. Accordingly, it has been established that the protein cross-section measures, in monomeric form, 20.52 nm^2. This is consistent with the value of 22 nm^2 previously reported [26]. We therefore conclude that our unit-cell should, at this point, be interpreted as a dimer of LacY.

Current studies are underway in our laboratory to improve the quality of high-resolution images presented here. Besides for a better comparison with the previously published 2D [9] and 3D [7] results, we are conducting 2D crystallization trials with the "red permease" and the C154G mutant.

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