Relaxation Processes in the Adsorption of Surface Layer Proteins to Lipid Membranes

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · NOVEMBER 2010

Impact Factor: 3.3 · DOI: 10.1021/jp107062e · Source: PubMed

Relaxation Processes in the Adsorption of Surface Layer Proteins to Lipid Membranes

Axel Hollmann,[†] Lucrecia Delfederico,[†] Graciela De Antoni,[‡] Liliana Semorile,[†] and Edgardo Aníbal Disalvo*[,§]

Laboratorio de Microbiología Molecular, DCyT, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, Bernal, Argentina; Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), FCEyN, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina; Laboratorio de Fisicoquímica de Membranas Lipídicas y Liposomas, Cátedra de Química General e Inorgánica, FFyB, UBA ciudad de Buenos Aires, Buenos Aires, Argentina

Received: July 28, 2010; Revised Manuscript Received: October 24, 2010

The present work evaluates the kinetics of the interaction of S-layer protein from *Lactobacillus brevis* with lipid monolayers by measuring the changes in the surface pressure as a function of time for different lipid compositions and at different lateral compressions. At high surface pressures, or at high cholesterol ratios, in which membrane rigidity and surface polarity are increased, the kinetics can be described by a pure diffusional process. At low pressures or in the absence of cholesterol, the kinetics of protein interaction can be interpreted as a consequence of a relaxation process of the membrane structure coupled to diffusion. As the less packed monolayers are more hydrated, the relaxation processes at low initial surface pressures could be ascribed to changes in water organization in the membrane. These observations denote that kinetic insertion of proteins can be modulated by components that modify the hydration state of the interface.

Introduction

The interphase of a lipid membrane is a meshlike structure where lipid polar head groups are imbibed in water.[1] This region is a kinetic barrier, in series to that composed by the hydrophobic core, for permeability of polar solutes and insertion of macromolecules. In addition, it has also been reported that water may penetrate into the hydrocarbon region of the lipid membrane.[2]

The interaction of proteins from the subphase with lipid monolayers is reflected by an increase in the surface pressure.[3−5] Upon injection of a soluble protein in the aqueous subphase of a lipid monolayer stabilized at an initial surface pressure Π_0, an increase in the surface pressure is observed in time until a stable value is reached. A criterion is to add the protein to the subphase of monolayers with a surface pressure above the surface pressure that corresponds to protein in water without lipids in the surface. In this condition, the changes in surface pressure of the monolayer observed upon the injection of the protein can be ascribed to an effect of the protein on the monolayer interfacial tension and not to effects of proteins on free-lipid spaces in the water subphase. This is usually fulfilled for surface pressures above 15−20 mN/m and, in these cases, the increase in surface pressure plotted for initial surface pressures of the monolayer usually gives a straight line with a negative slope. At surface pressures below 15 mN/m, the increase in pressure gives an increase in the surface pressure change. The reason for this behavior is beyond the scope of this paper and will be further discussed elsewhere.[6]

In previous papers, it has been shown that aqueous soluble proteins such as an aspartic protease show $\Delta\Pi$ vs Π_0 curves in which the limit pressure at which no further effect of protein on surface pressure was observed was dependent on the nature of the polar headgroup.[5] For instance, the limit pressure (or cutoff pressure) was much lower for dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) than for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) monolayers at the same temperature. This was consistent with the well-known fact that PE headgroup interactions are much stronger than those in PC due to the formation of intermolecular hydrogen.[7,8]

In addition, for a given headgroup the $\Delta\Pi$ vs Π_0 slope was dependent on the unsaturated−saturated ratio of hydrocarbon tails. These results were interpreted in terms of the presence of hydration water around the polar head groups and to confined water in between the hydrocarbon chains.[5] These two kinds of water would have relevance in relation to the different polarity and hydrophobicity of the proteins interacting with the lipids.

Thus, the interaction of water-soluble proteins, followed by the changes in the surface pressure, may be a combined consequence of effects on the interphase and the hydrocarbon core region. The identification of the membrane region involved is desirable from both the thermodynamic and structural viewpoints. The kinetics of interaction and final values of surface pressure of the protein adsorption in these conditions could provide an insight on the relevance of each region in the overall process.

S-layer proteins are one of the outer surface components of cell envelopes of many prokaryotic organisms, archaea, and bacteria. These proteins have the ability of self-assembly "in vitro", either in suspension or at liquid surface interfaces or on lipid films including liposomes.[9] In previous works, we showed that S-layer protein from *Lactobacillus brevis* are able to cover and stabilize liposomes composed of lecithin−cholesterol and stearylamine.[10,11] Moreover, the interest in S-layer lactobacilli has been reinforced by claimed and demonstrated probiotic properties for human and animal consumers.[12,13] Thus, it was of interest to analyze the kinetics of interaction of S-layers proteins with monolayers at different pressures and lipid composition.

* To whom correspondence should be addressed. Fax: 54-11 4508-3645. E-mail: eadisal@yahoo.com.ar.
[†] Universidad Nacional de Quilmes.
[‡] Universidad Nacional de La Plata.
[§] Universidad de Buenos Aires (UBA).

10.1021/jp107062e
Published on Web 11/18/2010

On this basis, the present work studies the interactions of S-layer proteins from *L. brevis* with lipid monolayers composed of phosphatidylcholines, cholesterol, and stearylamine spread at the air−water interface at different surface pressures. This composition was chosen because in previous works it was used for the preparation of S-layer-covered liposomes for studies as vaccine delivery vehicle.[10,11] The interest to have an insight on the lipid phase state and its consequence on the protein stabilization makes important the understanding of the kinetics and mechanism of adsorption of the S-protein for the preparation and stability of the products.

In this context, the surface activity and the affinity of S-layer proteins were evaluated at different initial lipid monolayer surface pressures (Π_0) by the surface pressure increase ($\Delta\Pi = \Pi - \Pi_0$) at steady-state conditions upon injection of the protein in the subphase. The kinetics of adsorption and penetration of the protein in PC monolayers was studied varying chain packing and the surface properties by the addition of cholesterol and charged lipids, respectively.

Experimental Methods

Lipids and Chemicals. Soybean phosphatidylcholine (PC), stearylamine (SA), and cholesterol were purchased from Sigma (St. Louis, MO). The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) were obtained from Invitrogen (Eugene, OR). Chloroform used was analytical grade and water was Milli-Q quality.

Bacterial Strains, Growth Conditions, and Isolation of the S-Layer Proteins. *Lactobacillus brevis* JCM 1059 was grown to midlog phase in 250 mL of MRS broth[14] (Biokar Diagnostics, Beauvais, France) at 37 °C, harvested by centrifugation (5.000*g*, 15 min, 4 °C), and washed twice in physiologic solution (NaCl 0.9% w/v). The S-layer proteins were extracted with LiCl 5 M (Carlo Erba, Milan, Italy) at 20 °C for 2 h. LiCl-extracted S-layer proteins were dialyzed against distilled water at 22 °C for 2 h under agitation. To ensure the absence of large S-layer aggregates, a centrifugation at 16.000*g* for 15 min at 4 °C was carried out by a modification of Jahn-Schmid et al. protocol.[15] The S-layer protein content of the clear supernatant was evaluated by SDS-PAGE 12.5%, and its concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, CA). SDS-PAGE analysis of LiCl-extracted S-layer proteins from *L. brevis* showed only a single protein band with an apparent molecular mass of 49.5, as published in a previous work.[10]

Changes on the Surface Pressure of Lipid Monolayers. The changes of the surface pressure of monolayers induced by S-layer proteins were measured in a Kibron μTrough S equipment (Kibron Inc., Espoo, Finland) at constant temperature (22 ± 0.5 °C). The surface of an aqueous solution contained in a Teflon trough of fixed area was exhaustively cleaned. Then, a chloroform solution of lipids was spread on this surface to reach surface pressures between 20 and 42 mN/m. At each chosen surface pressure, protein solutions were injected in the subphase and the changes on the surface pressure were followed during time to reach a constant value. The same procedure was followed for all monolayer compositions. Surface pressures at constant surface area were automatically recorded.

Preparation of Liposomes with DPH or Laurdan. Positively charged liposomes were prepared from a mixture composed of different soybean phosphatidylcholine (PC), cholesterol, and stearylamine (SA) ratios. Lipids were dissolved in 2 mL of chloroform and mixed with hydrophobic probes to achieve a final probe concentration of 0.33 mol % of Laurdan or DPH.

The chloroform solutions were evaporated under nitrogen flow to eliminate solvent traces. The dry lipid film was rehydrated by addition of 1 mL of H_2O and agitation above the stearylamine transition temperature (45 °C) for 1 h. After addition of the fluorescent probes, all samples were wrapped in aluminum foil to avoid fluorescence extinction.

Steady-State Anisotropy Measurement with DPH. The fluorescent lipophilic molecule DPH partitions in the hydrophobic region in bilayer,[16] and it has been often used as a probe to detect the gel or fluid state of the hydrocarbon core of lipid bilayer of liposomes, biological membranes, and whole cells, by monitoring the anisotropy $\langle r \rangle$ of its fluorescence.[17,18]

Measurements were done in a Perkin-Elmer Luminescence spectrometer model LS 55, equipped with excitation and emission polarizers and a circulating water bath. The temperature was controlled inside the cuvette with a thermocouple within ±0.2 °C. Steady-state anisotropy $\langle r \rangle$ was calculated by the following equation

$$\langle r \rangle = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

where I_{vv} and I_{vh} represent the fluorescence intensity obtained with the vertical and horizontal orientations of the excitation and emission polarizers. $G = I_{hv}/I_{hh}$ is a correction factor accounting for the polarization bias in the detection system.

Generalized Polarization Measurement with Laurdan. This method is based on the bilayer-order-dependent fluorescence spectral shift of Laurdan which can be attributed to dipolar relaxation phenomena, originating from the sensitivity of the probe to the polarity of its environment.[19] Laurdan is located at the hydrophilic/hydrophobic interface of the membrane bilayer with the lauric acid tail anchored in the phospholipid acyl chain region.[19,20]

Measurements and temperature controls were done as described above for steady-state emission intensity. The molar relation 1:300 Laurdan/lipid was chosen to increase the sensitivity of measurements without perturbing the lipid properties.[19,21]

Samples were excited at 350 nm and emission intensity was acquired for several hundred seconds at 435 (I_{435}) and 500 (I_{500}) nm. Generalized polarization (GP) was calculated from the emission intensities using the following equation adapted from the work of Parasassi et al.:[19]

$$\mathrm{GP} = \frac{I_{435} - I_{500}}{I_{435} + I_{500}} \quad (2)$$

***n* Coefficient Determination.** From eq 3

$$\Delta\Pi \cong k \times t^n \quad (3)$$

where k and n are the constants.[22] Regression analysis[23] from the $\Delta\Pi$ vs time media curves was performed to determine the values of n with Microsoft Excel by minimizing the root mean square error and chi-square error between the experimental data rate and the model equation.

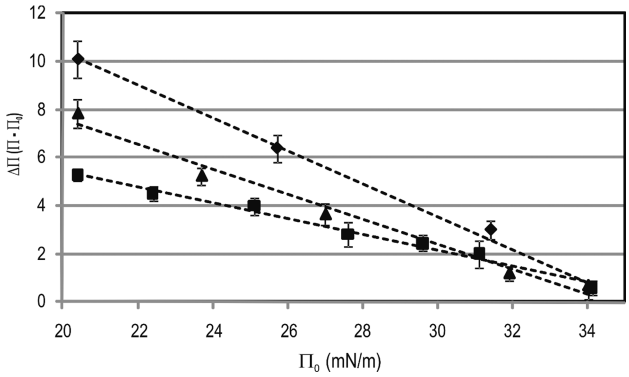

Figure 1. Changes in surface pressure as a function of Π_0 after addition of 0.90 μmol of S-layer protein from *L. brevis* on monolayer with soybean phosphatidylcholine, cholesterol, and SA in the molar relation of 10:5:1 (■), 10:2.5:1 (▲), and 10:0:1 (◆). Each point is the average of at least triplicates of independent samples.

Results

The interfacial tension of an air−water interface of a solution containing 285 μM of S-layer proteins from *L. brevis* is reduced with respect to pure water and the final surface pressure is around 18.0 mN/m (data not shown). For this reason, the lowest initial surface pressure of the lipid monolayers before the addition of the protein to the subphase was above that value. In this condition, the changes in surface pressure observed upon the injection of the protein can be ascribed to an effect of the protein on the monolayer interfacial tension.

The injections of increasing amounts of S-layer proteins in the subphase underneath monolayers composed of phosphatidylcholine, cholesterol, and stearylamine (10:5:1) at the initial surface pressure of 22 mN/m resulted in a subsequent increase of the surface pressure to reach a plateau of 27.1 mN/m around 1.8 μmol (250 μM) (data not shown). Therefore, for further analysis, a protein concentration corresponding to half of that saturation value was chosen as the test one to determine the effect of the surface pressure changes on monolayer response.

In this condition, the slope of the $\Delta\Pi$ vs Π_0 curves decreased when the cholesterol ratio in the monolayer was increased (Figure 1). The cutoff value of the three curves was around 35.3 mN/m with a standard deviation lower than 0.8 mN/m which is below the standard error. So they can be considered similar. Thus, as found for other aqueous soluble proteins,[5] the slopes of the curves appear related with the hydrocarbon core phase state and the cutoff with the nature of the polar headgroup. The lipid packing in a monolayer can be modified by applying a lateral pressure. In a bilayer, in which the lateral pressure cannot be controlled, it can be modified by the composition, via the chain or polar head groups lateral interactions.

Cholesterol increase in fluid membrane increases the packing producing a liquid condensed state in monolayers.[24,25] In contrast, the addition of charged components, in our case stearylamine, produces a decrease in packing due to the repulsion at the membrane surface.

Therefore, the correlation of the effects of cholesterol on the hydrocarbon phase state of a bilayer by measuring anisotropy ($\langle r \rangle$) and on the interphase by measuring the generalized polarization (GP) on liposomes with the surface changes induced by the protein in monolayers with the same cholesterol ratios can be useful to understand the influence of lipid composition on the surface pressure rate changes.

Anisotropy is a measure of the organization of the lipid chains when it is determined by a fluorophore partitioning into the hydrocarbon core. The degree of freedom of DPH is reduced to two dimensions when rigidity increases. In comparison to monolayers, the packing of the chains when lateral pressure is increased is equivalent to the anisotropy increase induced by the condensation of the chain in the gel state or by the addition of cholesterol.

As observed in Table 1, both anisotropy and GP increased with cholesterol ratio, an effect that can be ascribed to the condensing effect promoted by cholesterol on PC bilayers. In this regard, when the chains are ordered, the head groups' packing increases affecting water penetration at the head-group region of membrane phospholipids[20,21] reducing the number of solvent molecules. The values of GP decreased with the decrease in anisotropy due to the cholesterol depletion (Table 1), denoting that the chain packing also affects the interface region. However, these changes are not visible in the cutoff value as measured in monolayers.

The adsorption of proteins to a fluid interface trends to the reduction of interfacial tension. This behavior has been observed with different proteins on oil−water interphases. As $\Pi = \gamma^\circ - \gamma_{(t)}$, γ° being the surface tension of the pure lipid monolayer at a given surface pressure and $\gamma_{(t)}$ the interfacial tension change observed at different times after protein injection, the determination of the surface pressure as a function of time for different initial surface pressures indicates that proteins adsorb to the lipid membrane by reducing the interfacial tension in different stages.

According to the data presented above, the increase in surface pressure of the monolayer is a specular image of the decrease of the interfacial tension of the monolayer.

With the information provided by Figure 1, the kinetics of protein interaction with monolayers with different cholesterol ratio was compared. In the absence or at low cholesterol content, i.e., membranes at low packing, the kinetic pattern reflects a deviation from the linear behavior in the plot of square root of time. At high cholesterol ratios (high packing), when the rigidity and the polarity (Table 1) were above 0.121 ± 0.0089 and -0.032 ± 0.006, respectively, the deviation from the linear behavior became less noticeable (Figure 2). Thus, surface pressure increase during protein adsorption follows different time regimes at a constant protein concentration depending on the cholesterol ratio in the monolayer.

In order to gain some insight on the meaning of the cutoff and its relevance to the different time regimes in the adsorption kinetics, the changes of the $\Delta\Pi$ vs Π_o curves and the kinetics were studied varying the surface charges (SA amount) for a

TABLE 1: Anisotropy ($\langle r \rangle$) and Generalized Polarization (GP) Values of Liposomes, Slopes (m) of $\Delta\Pi$ vs Π Curves, and n Values Obtained from Kinetic Curves after Injection of S-Layer Protein on Lipids Monolayers[a]

lipid composition	$\langle r \rangle$	GP	m	n
soybean phosphatidylcholine and cholesterol (10:5)	0.191 ± 0.0029	0.494 ± 0.019	-0.326 ± 0.051	0.59
soybean phosphatidylcholine cholesterol, SA (10:5:1)	0.185 ± 0.0043	0.468 ± 0.032	-0.328 ± 0.027	0.70
soybean phosphatidylcholine cholesterol, SA (10:2.5:1)	0.173 ± 0.0054	0.167 ± 0.034	-0.519 ± 0.039	0.76
soybean phosphatidylcholine and SA (10:1)	0.121 ± 0.0089	-0.032 ± 0.006	-0.685 ± 0.041	0.85

[a] All measures were made at 22 °C.

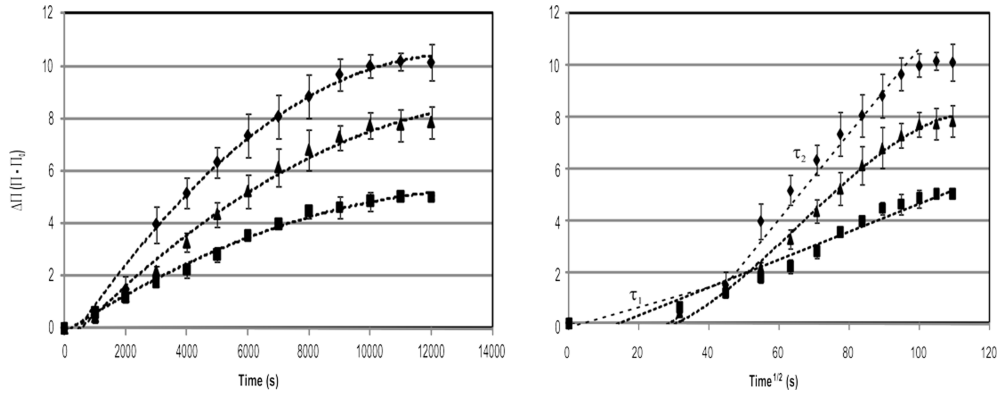

Figure 2. Variation of surface pressure after injection of 0.90 μmol of S-layer proteins from *L. brevis* on monolayer with soybean phosphatidylcholine, cholesterol, and SA in the molar relation of 10:5:1 (■), 10:2.5:1 (▲), and 10:0:1 (◆) as a function of time expressing in seconds (left) or or time$^{1/2}$ (right). Initial surface pressure for all curves was 20.5 mN/m. Each point is the average of at least triplicates of independent samples.

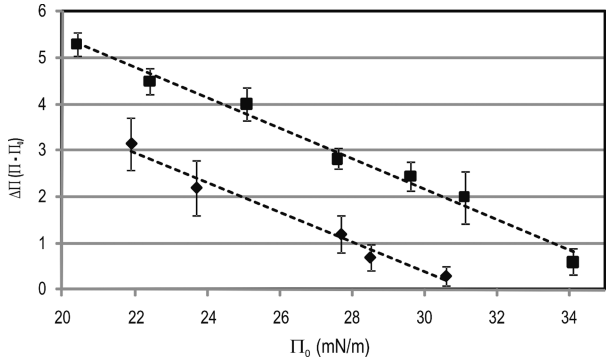

Figure 3. Changes in surface pressure as a function of Π_0 after addition of 0.90 μmol of S-layer protein from *L. brevis* on monolayer with soybean phosphatidylcholine, cholesterol, and stearylamine in the molar relation of 10:5:1 (■) or soybean phosphatidylcholine and cholesterol in the molar relation of 10:5 (◆). Each point is the average of at least triplicates of independent samples.

constant PC:cholesterol ratio. As stated above, the presence of surface charges induces a less rigid state due to the repulsions.

It was observed that the cutoff shifted from 35.3 ± 0.8 to 31.2 ± 0.18 mN/m after SA depletion remaining the slope unaltered (Figure 3). This effect is similar to that found for other proteins when PC monolayers were replaced by PE monolayers, denoting that lower cutoffs correspond with more packed states.[5]

The kinetic analysis shows that, at low initial pressures (22 mN/m) in the presence of stearylamine, S-layer protein adsorbs to monolayers showing a deviation from the linear behavior obtained when stearylamine content is depleted (Figure 4). In coincidence with the condensing effects of cholesterol and its correlation with the kinetic behavior, the anisotropy values on liposomes without SA, in which the kinetics is linear, were around 0.191 ± 0.0029, which is the highest value achieved in our experimental conditions (Table 1).

To confirm the relation between the kinetic patterns and the packing state of the monolayer, the evolution in time of surface pressure was measured in monolayers of a given composition (PC, cholesterol, and SA, 10:5:1) at low initial surface pressure (20.5 mN/m) and at high initial surface pressure (32 mN/m) (Figure 5). The adsorption kinetics at low pressures deviate from the linear behavior in comparison to that found at high pressure, confirming that kinetic regime is closely related to the packing states of the membranes.

In summary, when the packing of a monolayer was increased, either by lateral compression or by changing the lipid composition, the kinetics behavior is linear in terms of square root of time. Deviation of this behavior is consistently found in all cases when the packing was decreased.

Discussion

The increase of the ΔΠ values, as a measure of the response of the monolayer to protein interaction for a given initial surface pressure, follows the sequence (PC:SA) > (PC:SA:Chol) > (PC:Chol). This same sequence is found when anisotropy and GP

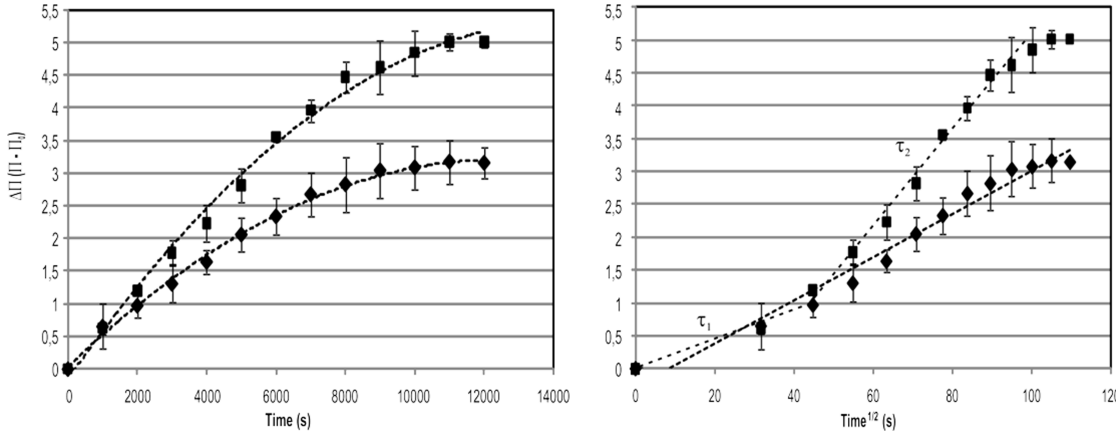

Figure 4. Variation of surface pressure after injection of 0.90 μmol of S-layer proteins from *L. brevis* on monolayers with soybean phosphatidylcholine, cholesterol, and SA (■) and without stearylamine (◆) as a function of time expressed in seconds or time$^{1/2}$. Initial surface pressure for both curves was 22 mN/m. Each point is the average of at least triplicates of independent samples.

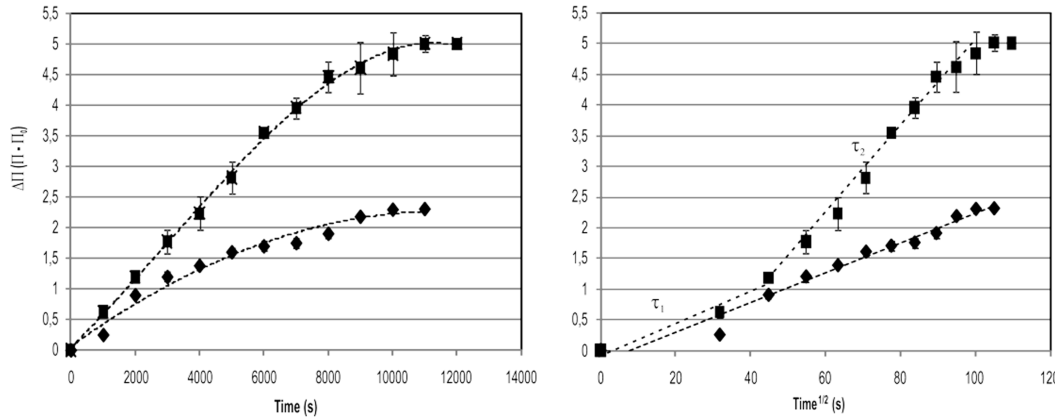

Figure 5. Variation of surface pressure after injection of 0.90 μmol of S-layer proteins from *L. brevis* on monolayers with soybean phosphatidylcholine, cholesterol, and SA when the initial pressure of monolayer was 22 mN/m (■) or 31.5 mN/m (◆), as a function of time expressed in seconds or time$^{1/2}$. Each point is the average of at least triplicates of independent samples.

are measured in bilayers. Thus, the increase in $\Delta\Pi$ in monolayers is correlated with the packing resulting from the combination of different components.

In PC:SA, the repulsion of charges makes the membrane less rigid and with the highest $\Delta\Pi$. The addition of cholesterol to a PC:SA membrane increases the rigidity with a parallel decrease in $\Delta\Pi$. Finally, in SA-depleted PC:cholesterol membranes, the rigidity is the highest and the $\Delta\Pi$ the lowest.

The changes in the packing are also reflected in the kinetic response of the membrane after protein addition (Figures 2 and 4). In the presence of cholesterol and the absence of SA the kinetics can be represented by a linear plot of the square root of time. The depletion of cholesterol or the addition of SA, both resulting in less rigid membranes, promotes a deviation from the linear behavior. The adsorption kinetics reported for S-layer proteins in this work follow a characteristic behavior observed for other proteins in oil−water and air−water interphases.[26−29] These authors ascribed different kinetic patterns to several conformational changes of the protein structure with time that may be reversible or irreversible, and proposed different expressions for adsorption of proteins to solid and liquid interfaces.[26−30] All of them consider the mechanism of adsorption similar to that of particles due to the protein complexity. In our case, we will not consider details of the protein structure and will focus on the effects that proteins induce in the lipid interface and the variations that changes in the surface lipid properties may have on the adsorption kinetics Then, studies of a single protein varying the lipid matrix can be interpreted in terms of changes in the lipid surface depending on its composition and state of packing.

For a better comparison between the different deviations in the kinetic analysis with the phase state of the lipid interphase, a coefficient n is defined according to eq 3. The response of the monolayer to protein interaction as a function of the time (t) can be described by

$$\Delta\Pi \cong k \times t^n \tag{3}$$

where, according with the different models used by different authors to describe the kinetics of protein adsorption, k includes the diffusion coefficient of the protein and the concentration of protein in the bulk which is constant in our case.

Lipid membranes in the rigid state present a linear regime during the whole time window (Figures 2, 4, and 5). The effects of diffusion on surface concentration of an adsorbing species ($\Gamma_{(t)}$) is predicted using the equation of Ward and Tordai.[29]

$$\Gamma_{(t)} = 2\frac{C_b}{\pi^{1/2}}D^{1/2}t^{1/2} \tag{4}$$

where $\Gamma_{(t)}$ is the interfacial concentration, C_b is the bulk protein concentration, and D is the diffusion coefficient.

As the surface pressure can be related to the surface concentration (Γ) by

$$\Pi = \Gamma RT$$

where R is the gas constant and T is temperature in kelvin, the change in surface pressure can be expressed as a solution of the second Fick's law

$$\Pi = \frac{2RTC_b}{\pi^{1/2}}D^{1/2}t^{1/2} \tag{5}$$

The plot of $\Delta\Pi$ vs $t^{1/2}$ according to eq 3 and considering eq 5 permits to calculate the diffusion coefficient for a given protein concentration ($C_b = 125$ μM) in bulk and the temperature $T =$ 294.6 K which gives $D = 6.93 \times 10^{-7}$ cm^2/s for the monolayer of PC:Chol (10:5) without SA at the initial pressure of 22.0 mN/m (Figure 3) and $D = 3.69 \times 10^{-7}$ cm^2/s for the monolayer with PC:Chol:SA (10:5:1) at 31.5 mN/m (Figure 5). These values are in agreement with diffusion coefficient of several proteins reported elsewhere.[29]

For membrane at low lateral pressures or in an expanded state due to the presence of SA or the depletion of cholesterol, at least two regimes can be noticed. In some cases, the regime at initial times shows a much lower slope than that in rigid membranes, i.e., lower diffusion coefficient as compared to those stated above. That is, the rate of adsorption is very low, or it does not affect the surface pressure.

At a longer time, surface pressure slope increases, denoting a change in the diffusion coefficient. This observation indicates that there are, at least, two regimes of diffusion detectable within the experimental error suggesting that the protein adsorption changes the diffusing matrix with time. The lines in the Figures 2, 4, and 5 are drawn to highlight the different trends.

The deviation of a diffusive adsorption process can also be discussed by models which involve one or two relaxations times which could explain the linear deviation.

Klebanau et al.[30] have proposed that changes in surface pressures can be explained by two different relaxation times (eq 6):

$$\frac{\Pi_{(t)} - \Pi_\infty}{\Pi_0 - \Pi_\infty} = A_0 \exp(\frac{-t}{\tau_1}) + A_1 \exp(\frac{-t}{\tau_2}) \qquad (6)$$

where A_0, A_1, τ_1, and τ_2 reflect the different regions of time dependence of Figures 2, 4, and 5.[30]

The fitting by eq 6 of the data corresponding for the monolayer with PC and SA (10:1) without cholesterol (Figure 2) for which the deviation of linearity is the highest ($n = 0.85$) gives $A_0 = 1.003$; $\tau_1 = 7815.6$ s; $A_1 = 1.61$, $\tau_2 = 3244.4$ s. This observation denotes that there are at least two regimes of diffusion, implying that protein adsorption changes the diffusing matrix with time.

It is interesting to observe that when the deviation is less noticeable ($n = 0.7$ for PC:Chol:SA, 10:5:1 at low pressures, Figures 4 and 5) the time constants are much closer ($A_0 = 0.997$; $\tau_1 = 4539.6$ and $A_1 = 1.096$; $\tau_2 = 4172.9$).

At short times, protein at the water−membrane interface may exhibit reversible adsorption to a large extent. At longer times, hydrophobic regions may be exposed and protein may have a greater affinity for the membrane interface and be adsorbed more strongly than at the earlier stages. This effect is probably mediated by both surface hydrophobicity and conformational changes of the groups at the membrane interface and water reorganization.

In conditions in which the lateral pressure is high, the kinetics is similar to that obtained with high cholesterol ratio, which is known to increase the membrane rigidity. In both cases, the rate of adsorption follows that derived from the second Fick's law by which the amount of adsorbed protein is linear with the square root of time. This behavior is similar to that found for the adsorption of protein to oil−water interphase at low concentrations and molecules adsorb irreversible by diffusion. This means that the binding to the surface is fast.

When the surface layer becomes saturated, the adsorption rate decreases because it is no longer irreversible. That means that protein arriving at the interphase does not interact with the lipid surface and therefore they accumulate in the layers near the membrane. In other words, the protein does not find enough sites to interact. In this condition, the process may continue if the amount of protein in the adjacencies affects the lipid surface opening new sites. In this condition, the diffusional process is coupled to a relaxation due to the reorganization of the lipid interphase. The creation of new sites can be interpreted as an activated process in which rearrangement of the cross-linking network in the lipid surface (probably H bonds) is disrupted and re-formed by the protein insertion.

The n values obtained after fitting the curves for the different compositions are shown in Table 1, calculated by eq 3. It is observed that n values higher than 0.7 correlate with a significant decrease in membrane rigidity and polarity. However, in the range 0.6−0.7 the anisotropy and GP values remain nearly constant, indicating that other properties of the membrane besides rigidity and polarity are influencing the kinetic of protein interaction.

It is interesting to observe that the lowest n value corresponds with a membrane in which no net charges are present (PC: cholesterol mixture without SA).

Another indication that the kinetics pattern described by the n value is related to the compression of the membrane is given by the response to the protein interaction of monolayers at low and high pressures. At low surface pressures, PC:SA:Chol monolayers show a deviation of n to higher values (0.70), while at high pressures (0.61) the same monolayer shows a more linear plot (Figure 5).

A physical interpretation of the n value can be discussed in terms of normal and anomalous diffusion. The linearity of the amount of substance sorbed by a material with the square root of time assumes that the system responds to a linear gradient of concentration across the material in a steady state. In this case the surface concentration of the substance on the membrane is maintained constant, i.e., no accumulation of substance is produced during the transport which means that the material is homogeneous. This behavior has been also described as a nonobstacle diffusion process.[31,32]

When the diffusion behavior cannot be described adequately by this concentration dependence for Fick's law, anomalous or non-Fickean diffusion occurs. The essential distinction is that the adsorbent material responds rapidly to changes in their conditions. Anomalous effects may be directly related to changes in the surface concentration that may account for changes in the solubility and diffusional mobility. This may occur if the process of adsorption triggers a change in the surface that may result from the internal stress exerted by one part of the medium on another as the insertion proceeds. This is also described as obstacle diffusion; i.e., diffusion coefficient is not constant along the process.

This increase of cholesterol gives place to a kinetics behavior that can be described as a pseudo-Fickean one. This term has been used to describe sorption curves of the same general shape as in the Fickean behavior but for which the initial linear portion persists for a shorter time. When cholesterol is depleted and SA is added, a sigmoid-shaped curve is observed which would correspond to an anomalous diffusion. The Fickean process appears in packed membranes in which water arrangements are difficult to take place.

Cholesterol depresses the enthalpy of the phase transition that involves drastic changes in hydration. Thus, changes in the cholesterol ratio may affect the water levels in the membrane, as it seems to be confirmed by the increase in GP values. In membranes with lower cholesterol ratios, for which higher water content is expected, the adsorption process deviates from the Fickean behavior. In this condition, if the interaction of the protein is taking place in relation to a lyotropic transition, the absence of cholesterol can affect the protein adsorption by means of a dehydration process. In fact, the value of GP increase with the addition of the protein to PC:SA:Chol membranes.[11]

When the monolayer is compressed, the dehydration occurs by the packing increase independent of cholesterol, and thus the adsorption $\Delta\Pi$ vs Π_0 converges to a similar cutoff (Figure 1). Protein interaction kinetics seems to be influenced by the change in hydration state of the membrane induced by cholesterol.

Based on literature data,[8,33,34] it can be stated that the coexistence of opposing effects leads to hydration and dehydration of the membrane when compounds interact with lipid interfaces. Enhanced hydration results from the reorientation of tilted head-group orientation and aliphatic chain order leading to additional water binding sites and increase of water-accessible surface area. Dehydration is explained by the replacement of water at water binding sites. Considering the fact that lipid hydration is essentially driven by entropic phenomena, such as the increase of motional freedoms of phospholipids at endo-

thermic hydration,[33,34] the conclusion is that the non-Fickean diffusion involves a disruption of the meshlike structure in the interphase region. In this particular system, S-layer proteins-PC based monolayers, it was previously showed that the attachment of S-layer proteins on the lipid membranes is able to induce some changes in the lipid phase of membranes.[11]

The results also allow a better understanding of interactions involved in S-layer-lipid membranes. As we described above, protein adsorption seems to be dependent on the surface charges displayed by the presence of SA. Depletion of SA promotes a shift of the cutoff to lower values, while the slope remains unchanged for a given cholesterol−PC ratio. That is, the limit of surface pressure at which protein cannot affect the monolayer is much lower in the absence of surface charges. This also reflects that tighter membranes are more difficult to achieve in the presence of SA due to the lateral electrostatic repulsion in the membrane plane. Congruently to the behavior described above, in this condition, proteins adsorb with a Fickean pattern.

In addition, the interaction of S-layer proteins, which are positively charged, with lipid neutral and charged membranes was previously demonstrated[10,11] to produce a drastic change in the membrane structure as derived from the non-Fickean process described by data in Figure 5. Interestingly, the absence of SA promotes a linearization of the square root time plot, meaning that the process turns into one in which pure diffusion prevails; i.e., no further changes in the structure is apparent. Taken together, charge screening of SA triggers changes in the hydrocarbon phase disregarding its composition in neutral species.

Therefore, surface charges appear to be an important structural factor for adsorption of S-layer proteins and stabilization at the membrane surface. Moreover, our data suggest also that SA appears to play a role in the synergistic response of the membrane packing and hydration contributing to the adsorption of proteins.

Previous reports showed that adsorption of this S-layer protein was favored in fluid membranes: S-layer proteins adsorb preferentially on liposomes composed of PC lecithin (liquid crystalline state) than DPPC (gel state) at 20 °C,[10] in good correlation with the data obtained in this work.

Conclusions

The kinetics of interaction in monolayers is sensitive to the surface pressure.

The lateral pressure, and therefore the kinetics of interaction, can be modulated by the components of the lipid membrane: cholesterol condensing the PC monolayer and surface charges promote an expansion of them. In monolayers at low surface pressure, the kinetics appears to include a process of structural relaxation. This process appears to be related to the access of water to the membrane interface.

Acknowledgment. This work was supported by grants from Universidad Nacional de Quilmes, Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA), and PICT 2003-0327. G.D.A and L.S. are members of the Research Career of CIC-PBA and E.A.D. is member of the Research Career of CONICET.

References and Notes

(1) Cevc, G. *J. Chem. Soc., Faraday Trans.* **1991**, *87*, 2733–2739.
(2) Simon, S. A.; McIntosh, T. J. *Methods Enzymol.* **1986**, *127*, 511–521.
(3) Borioli, G. A.; Caputto, B. L.; Maggio, B. *Biochem. Biophys. Res. Commun.* **2001**, *280*, 9–13.
(4) Le Bihan, T.; Pelletier, D.; Tancrède, P.; Heppell, B.; Chauvet, J. P.; Gicquaud, C. R. *J. Colloid Interface Sci.* **2005**, *288*, 88–96.
(5) Martini, M. F.; Disalvo, E. A. *Biochim. Biophys. Acta* **2007**, *1768*, 2541–2548.
(6) Lehtonen, J. Y.; Rytömaa, M.; Kinnunen, P. K. *Biophys. J.* **1996**, *70*, 2185–2194.
(7) Pink, D. A.; McNeil, S.; Quinn, B.; Zuckermann, M. J. *Biochim. Biophys. Acta* **1998**, *1368*, 289–305.
(8) Pohle, W.; Selle, C.; Fritzsche, H.; Bohl, M. *J. Mol. Struct.* **1997**, *408/409*, 273–277.
(9) Schuster, B.; Sleytr, U. B. *Rev. Mol. Biotechnol.* **2000**, *74*, 233–254.
(10) Hollmann, A.; Delfederico, L.; Glikmann, G.; De Antoni, G.; Semorile, L.; Disalvo, E. A. *Biochim. Biophys. Acta* **2007**, *1768*, 393–400.
(11) Hollmann, A.; Delfederico, L.; De Antoni, G.; Semorile, L.; Disalvo, E. A. *Colloids Surf., B Biointerfaces* **2010**, *79*, 191–197.
(12) Mercenier, A.; Pavan, S.; Pot, B. *Curr. Pharm. Des.* **2003**, *9*, 175–191.
(13) Bernardeau, M.; Vernoux, J. P.; Henri-Dubernet, S.; Guéguen, M. *Int. J. Food Microbiol.* **2008**, *126*, 278–285.
(14) DeMan, J. C.; Rogosa, M.; Sharpe, M. E. *J. Appl. Bacteriol.* **1960**, *23*, 130–135.
(15) Jahn-Schmid, B.; Graninger, M.; Glozik, M.; Küpcü, S.; Ebner, C.; Unger, F.M:; Sleytr, U. B.; Messner, P. *Immunotechnology* **1996**, *2*, 103–113.
(16) Parasassi, T.; Sapora, O.; Giusti, A. M.; Stasio, G. D.; Ravagnan, G. *Int. J. Radiat. Biol.* **1991**, *59*, 59–69.
(17) Shinitzky, M.; Barenholz, Y. *Biochim. Biophys. Acta* **1978**, *515*, 367–394.
(18) Lentz, B. R. *Chem. Phys. Lipids* **1989**, *50*, 171–199.
(19) Parasassi, T.; De Stasio, G.; Ravagnan, G.; Rusch, R. M.; Gratton, E. *Biophys. J.* **1991**, *60*, 179–189.
(20) Chong, P. L. G.; Wong, P. T. T. *Biochim. Biophys. Acta* **1993**, *1149*, 260–266.
(21) Lisi, A.; Pozzi, D.; Grimaldi, S. *Membr. Biochem.* **1993**, *10*, 203–212.
(22) Crank, J. *The mathematics of diffusion*; Oxford University Press: Oxford, UK, 1975.
(23) Montgomery, D. C.; Runger, G. C. *Applied Statistics and Probability for Engineers*; John Wiley & Sons: New York, 1999.
(24) Marsh, D. *Biochim. Biophys. Acta* **2010**, *1798*, 688–699.
(25) Marsh, D.; Smith, I. *Biochim. Biophys. Acta* **1973**, *298*, 133–144.
(26) Hansen, F. K.; Myrvold, R. J. *Colloid Interface Sci.* **1995**, *176*, 408–417.
(27) Pereira, L. G. C.; Theodoly, O.; Blanch, H. W.; Radke, C. J. *Langmuir* **2003**, *19*, 2349–2356.
(28) Freer, E. M.; Yim, K. S.; Fuller, G. G.; Radke, C. J. *Langmuir* **2004**, *20*, 10159–10167.
(29) Beverung, C. J.; Radke, C. J.; Blanch, H. W. *Biophys. Chem.* **1999**, *81*, 59–80.
(30) Klebanu, A.; Kliabanova, N.; Ortega, F.; Monroy, F.; Rubio, R. G.; Starov, V. *J. Phys. Chem. B* **2005**, *109*, 18316–18323.
(31) Ratto, T. V.; Longo, M. L. *Langmuir* **2003**, *19*, 1788–1793.
(32) Sanabria, H.; Kubota, Y.; Waxham, M. N. *Biophys. J.* **2007**, *92*, 313–322.
(33) Binder, H.; Kohlstrunk, B.; Heerklotz, H. H. *J. Colloid Interface Sci.* **1999**, *220*, 235–249.
(34) Binder, H.; Kohlstrunk, B.; Heerklotz, H. H. *Chem. Phys. Lett.* **1999**, *304*, 329–335.

JP107062E