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INTERACTION OF GLOBULAR PROTEINS WITH MIXED LIPID VESICLES
A thermodynamic study of the lipid lateral phase separation

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Vesicles of charged (Phosphatidic Acid) and neutral (Phosphatidylcholine) lipids were used as membranes model to examine the lateral phase separation induced by a globular protein, namely lysozyme.

The ability of the positively charged protein, adsorbed onto vesicles surfaces, to induce the formation of micro-domains richer in the charged lipid component has been investigated by calorimetric measurements, using a DSC instrument.

The protein adsorption, rate of denaturation and lipid micro-domains formation were affected by *pH* and salt concentration variations showing the deep influence of the electric charges in modulating this phenomena.Some of the present results have been rationalized on the basis of a theoretical model recently developed by the authors.

Introduction

Water-soluble proteins and pyroelectrolytes are known to influence the structure and function of biological membranes causing many structural changes, one of them being the segregation of different lipids (charged and neutral) into micro-domains. The formations of regions richer in one lipid component may influence several properties of biological membranes, such as membrane potential [1], rate of fusion between adjacent vesicles [2] and reactivity of intrinsic proteins inbedded in the bilayer [3]. Moreover, high local concentration of lipids having large headgroups could lead to the formation of non-bilayer structures within the flat vesicle (cell) membrane leading to a destabilization of the lipid assembly [4].

Another important and related effect is the strengthening of the soluble protein-lipid interactions due to the clustering of opposite charges at the binding site. On the other hand, the tighter lipid-protein binding may cause a larger span of the polymeric chain over the membrane plane, leading to con-

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formational variations of the protein [5]. Therefore, the protein binding constant, its conformational (shape) variations and the lipid redistribution at the binding site should be related in a self-consistent way.

Previous investigations reported in the literature suggest that many basic protein or polyaminoacids strongly interact with negatively charged bilayers forcing the clustering of charged lipids into the protein adsorption sites. Among the polypeptides showing a capability to segregate the membrane lipid component we recall the myelin basic protein [6] and polylysine [7], whereas other proteins do not seem particularly effective [8].

This problem has been addressed by us in a recent paper [9] where we developed a simple theoretical model to study a water-soluble globular protein interacting with a mixed lipid membrane containing two different lipids. The predictions of our theory (see below) were compared with some preliminary results obtained for the DPPC/DPPA/polylysine system.

We extend now our study to the DPPC/DPPA/lysozyme system at different ratios of charged/neutral lipids. This system allows us to investigate both the protein-induced lipids lateral phase separation and the related shifts of the protein unfolding temperature.

Theory

For sake of completeness, let us briefly recall the basic features of the theory developed in [9]. The surface of the lipid membrane has been treated as a two-dimensional random mixture of neutral and charged particles and the protein (or polymer), as a spherical deformable but incompressible 'drop of oil' bearing a given charge density over its surface. Some proteins can be adsorbed onto the lipid surface by deforming their shape to better interact with the charged lipid head groups. Also the lipid molecules can redistribute themselves within the bilayer in order to bind more favourably the protein charged residues. The number of adsorbed proteins, size of the membrane-protein contact region, shape of the adsorbed protein-water interface and lipid composition in the contact region, were considered as variational parameters and the total energy of the system (entropic and enthalpic contributions) was expressed as a function of these parameters.

The considered main energy contributions, evaluated both in protein-free and protein-bound regions of the bilayers, were:

a) mixing entropy of the two lipid components;

b) electrostatic repulsion between the lipid charged head groups;

c) adsorbed protein (polymer) membrane binding energy;

d) work associated to the surface deformation of the adsorbed protein (polymer);

e) entropic and enthalpic variations of the adsorbed proteins (polymers) on passing from the water solution (3D) to the membrane plane (2D);

Minimization of the total energy gave the searched values for all the variational parameters. In particular we obtained information on the lipid composition of the membrane regions in contact with the adsorbed proteins. Such an inhomogeneous distribution, where the charged lipids are clustered into micro-domains, causes the splitting of the excess heat capacity peak associated to the melting of the lipid hydrocarbon chains melting (the $L_\alpha \rightarrow L_\beta$ transition). An interesting prediction of our theory concerns the composition of the micro-domains as a function of the stoichiometric mole fraction of the two lipid components. The plot of the charged lipid excess concentration $X_1^B - X_1^A$ (defined as a difference of the charged lipid fraction between the protein-adsorbed and protein-free regions of the bilayer) *vs.* its stoichiometric concentration X_1 yields very asymmetric curves (see Fig. 1).

In more detail, the excess of charged lipid is zero for a one-component bilayer reaching a maximum at low charged lipids concentrations. The phenomenon is reduced when strong repulsions between the charged lipid

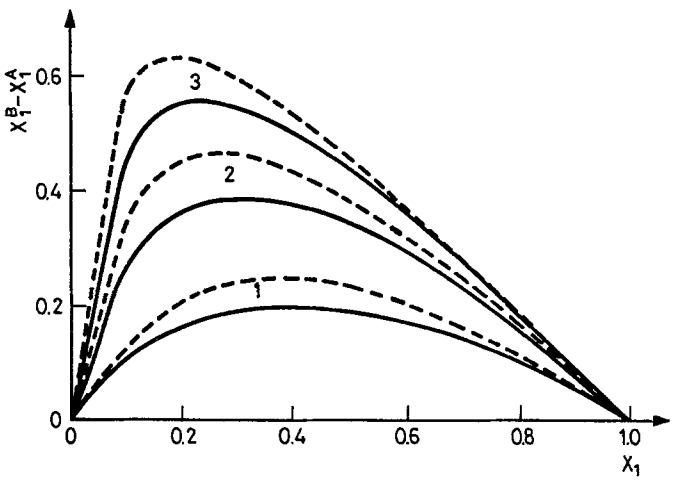

Fig. 1 Excess of charged lipids in the lipid-protein contact region *vs.* the molar fraction X_1 of the charged lipid component. The curves have been obtained for different values of the dimensionless lipid-protein interaction parameter $\rho/K_B T$($\rho/K_B T = 1$, 2 or 3) and head-group repulsion parameter $\tilde{\eta}/K_B T$ ($\tilde{\eta}/K_B T = 0$, dashed lines and $\tilde{\eta}/K_B T = 1$, full lines). K_B is the Boltzmann's constant, T the absolute temperature, ρ and are $\tilde{\eta}$ the head-head and protein-head energies per lipid molecule. The protein concentration was held constant in all calculations

head groups take place whereas it is enhanced by strong lipid-protein interactions (see Fig. 1) but the general shape of the curves remains unchanged by variation of those parameters.

An increased binding constant between lipid and protein and the enlarged lipid-protein contact area induced by the lipid lateral phase separation have been calculated in the framework of our theory [9] but the results obtained have not been yet experimentally tested. However, some indirect information could be obtained by analyzing the variations of the calorimetric peak associated to the protein denaturation, that, in some cases, can be very intense, reversible and well-separated from the peak related to the lipid $L_\alpha \rightarrow L_\beta$ transition.

Materials and methods

Synthetic $L-\alpha$-dipalmitoylphosphatidylcholine (DPPC) was obtained from Fluka (puriss.), synthetic $L-\alpha$-dipalmitoyl phosphatidic acid (DPPA) was obtained from Sigma. The purity of these samples has been checked by bidimensional thin layer chromatography. Phosphorous phospholipid's content was essayed as inorganic phosphate by the analytical procedures previously reported [10].

Lysozyme from Chicken egg white ($MW = 14.000$), grade I, 3 times crystallized, dialyzed and lyophilized was obtained from Sigma and used without further purification.

Lipids film lyophilized for 3 hours, mixtures or the pure lipids (with ca. 14 micromoles of total lipids), were added with 250 μl of buffered 50 mM Tris (pH 7), alone or containing lysozyme at a concentration of $3.6 \cdot 10^{-3} M$.

The ratio protein to lipids was $6.3 \cdot 10^{-2}$. The ionic strength, adjusted by NaCl was held constant at $5.5 \cdot 10^{-2}$ M. Some additional experiments were performed at higher ionic strength ($4.4 \cdot 10^{-1} M$).

The samples were vortexed twice for 1 min at 75° and then shaken for 3 hours at 70° in a water bath to homogenize the dispersion. Afterwards, aliquots of 120 μl of each sample, were transferred and sealed in aluminium pans. After he DSC run the phosphorous content of the lipidic samples was determined as above mentioned.

Differential scanning calorimetry was performed with a Mettler TA 3000 calorimeter, equipped with a DSC 30 cell and a TC 10 processor. The sensitivity used was 1.71 mW full-scale. The samples were analyzed by using a heating and cooling rates of 2 deg/min in the temperature range of 10–80°.

Results and discussion

In Figs 2*a* and 2*b* we report the DSC curves for different mixtures of charged and neutral phospholipids. Both phospholipids have the same hydrophobic tails (two palmitic acid chains) but different head groups: the charged phosphatidic acid (PA) (one negative charge at neutral *pH*s) and the neutral zwitterrionic choline (PC).

The curves reported in Fig. 2*a* refer to protein-free samples, whereas those reported in Fig. 2*b* refer to lysozyme containing lipid dispersions.

Lysozyme was chosen because it is a typical globular protein of modest size (129 amino acid residues) and its thermal unfolding is a simple two-state process in aqueous solution [11]. Its thermal denaturation is partially reversible [12] and occurs at quite high temperatures much over that of the gel to liquid crystal transition of saturated phospholipids.

Without lysozyme a complete mixing of the membrane lipid components, both in the gel and liquid crystal phases, is evident (Fig. 2*a*). When we add

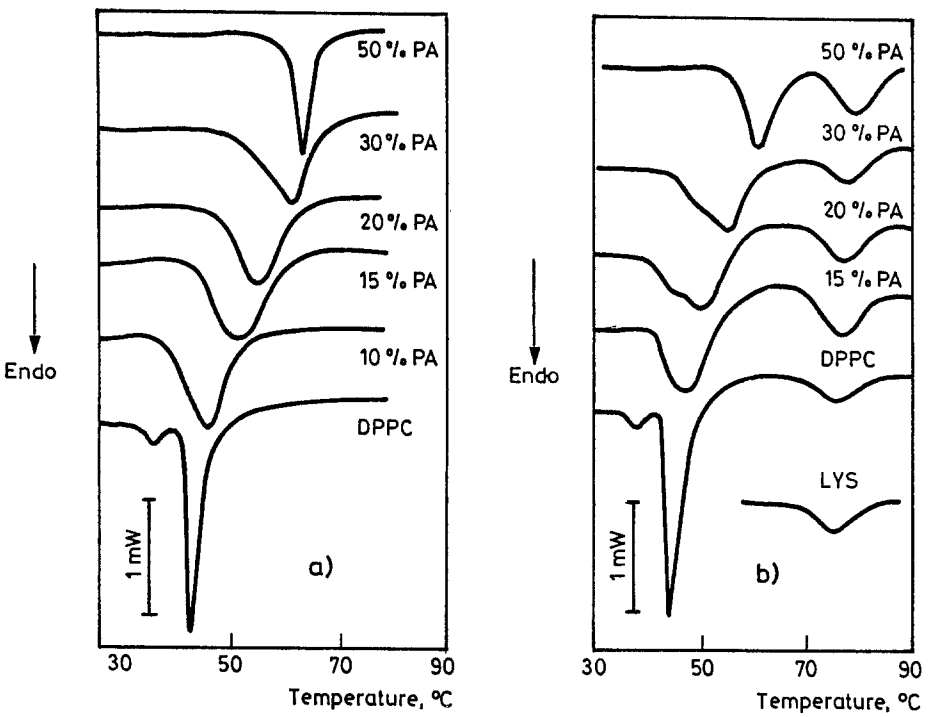

Fig. 2 Typical DSC heating curves of DPPC and DPPA (PA) liposomes with different molar ratio of the lipidic components. The liposomes have been suspended in Tris buffer (Fig. 2*a*) and in Tris buffer containing lysozyme with a protein/lipid molar ratio equal to $6 \cdot 10^{-2}$ (Fig. 2*b*)

the protein, a splitting of the peak, suggesting a lateral phase separation of the bilayer lipid components, is observed (Fig. 2*b*).

The splitting of the lower temperature peak, associated to the lipid phase transition, starts at low concentration of charged lipid ($X_1 \approx 15\%$), reaches a maximum when $X_1 \approx 20\%$ then rapidly decreases, disappearing when $X_1 > 30\%$.

This interesting behaviour is fully confirmed by our theory. In fact, since the peak splitting is proportional to the function $X_1^B - X_1^A$ (describing the excess of charged lipid in the lipid-protein contact region, see also Eq. (34) of [9]) we can obtain a direct comparison between the experimental and theoretical results. This can be done looking at Figs 1 and 2*b*. Indeed, in both cases the maximum of the peak splitting and that of the function $X_1^B - X_1^A$ take place at comparable low values of X_1. Furthermore, it is worth noting that our model not only correctly predicts the variation of the peak splitting *vs.* the charged lipid concentration, but also it gives the correct order of magnitude of the phenomenon. In fact, assuming the lipid-protein interaction to be in the range of 4 kJ/mole per lipid molecule and taking the same value for the repulsion parameter between the lipid head-groups, we calculated in [9] a T_m peak splitting of about 10°, in good agreement with the experimental findings. This result suggests that the protein-induced lateral phase separation can occur in a wide class of biological systems by spending a relatively small amount of energy to balance the entropy loss due to the lipids clustering.

However, it must be pointed out that our model is based on the assumption that the protein has a negligible effect on the thermotropic behaviour of one-component lipid membranes, the only effect of the protein-bilayer interaction being the lipid redistribution into micro-domains with different composition. This condition is fulfilled by hydrophilic proteins, whereas the occurrence of hydrophobic domains may perturb the lipid packing, making the interpretation of the thermotropic behaviour even more difficult. Preliminary measurements on pure DPPC and DPPA systems did not show significant ($\simeq 1^\circ$) shifts of $L_\alpha \rightarrow L_\beta$ transition temperatures.

In order to test our theory with further investigations, we preformed some DSC measurements in mixed lipid membranes at different values of basicity.

At these *pH* value the thermotropic behaviour of PCs is not affected as well as the charge born by the lysozyme basic residues remain constant at neutral or basic *pH*'s (isoelectric point = 11.1, ref. 6a). On the contrary, the acidic PAs bears two negative charges at basic *pH*'s and one at neutral *pH*'s [13], therefore, the electrostatic repulsion between the ionic lipid head-groups increases four times on doubling the head's charge.

According to our theory, this implies a lowering of the peak splitting magnitude and it is fully confirmed by DSC measurements which show an asymmetric unresolved band (see Fig. 3) instead of the two-peak structure reported in Fig. 2*b*.

However, these results can be considered only on a quantitative basis since doubling of the head-groups charge may cause other effects such as, for example, a tighter lipid-protein interaction.

Also the shift at the $L_\alpha \rightarrow L_\beta$ transition temperature toward higher figures is consistent with the hypothesis of a less tight packed lipid membrane and has been reported in previous papers [14].

On the contrary, lowering the *pH* (*pH* =3) the more favourable hydrogen-bonding interactions lead to lateral phase separation of the two lipids even in absence of protein (see Fig. 3). Protein addition makes the DSC run more complex, preserving the multi-peaks pattern.

In this study we extend our investigation also to the calorimetric peak related to protein unfolding.

The specific heat variations of vesicles-bound lysozyme are reported in Fig. 2*b*. As we can see, the interaction with neutral DPPC vesicles does not change the lysozyme thermotropic behaviour, whereas, on raising the amount of the charged DPPA component, the denaturation peak is shifted toward higher values, the unfolding enthalpy seems to be slightly increased and the peak becomes more asymmetrical.

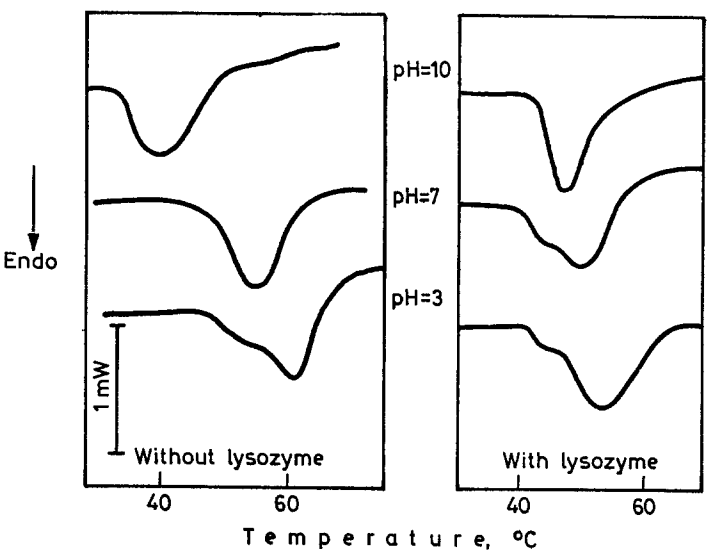

Fig. 3 Effect of *pH* on the lipid lateral phase separation of DPPC/DPPA (80:20) liposomes (Tris buffer, ionic strength $=5.5 \cdot 10^{-2}$ *M*)

These results show unambiguously a stronger lysozyme-charged vesicles interaction, which, however, does not increase dramatically on raising the amount of charged lipids.

An other interesting phenomenon was observed by us during some control experiments performed to check the constancy of the transition temperatures in subsequent heating-cooling cycles. Indeed, the intensity of the peak associated to the protein denaturation was progressively reducing by repeating the thermal cycles because of the partial reversibility of the lysozyme unfolding transition [12], however, when the protein was interacting with vesicles containing even a small amount of acidic lipids (less than 20% of DPPA), the rate of denaturation was reduced (see Fig. 4). This effect was not evidenced

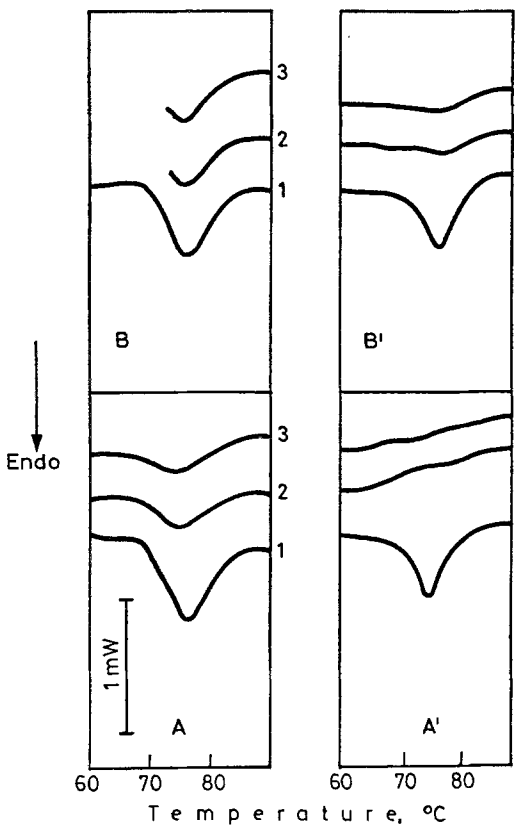

Fig. 4 Effect of protein-lipid interaction on the rate of protein denaturation at different ionic strength (curves *A*, *B* $=5.5 \cdot 10^{-2}$ *M* and curves *A'*, *B'* $=4.4 \cdot 10^{-1}$ *M*. DSC heating curves show the denaturation curves of lysozyme interacting with DPPC(*A*, *A'*) or DPPC/DPPA (80:20) (*B*, *B'*) liposomes suspended in Tris buffer (*pH* $=7$). The number on the right hand side of the curves refer to subsequent scans. The elapsing time between two heating scans was 1.5 hours

by using pure DPPC vesicles and is not enhanced appreciably by increasing the percentage of acidic lipids (data no reported here). This result is a further example of how the formation of lipid micro-domains provides a constant environment to the bound proteins. For example, recent studies performed by using a hydrophobic photolabel reacting with myelin basic protein, showed the same yield of labeled protein, either when the protein was bound to DPPA or DPPA/DPPC (1:1) vesicles, while the figures were much smaller for vesicles containing DPPC alone [15]. This fact further confirms that the clustering of charged lipids in the protein adsorption site makes the binding almost independent of the bilayer composition.

This study on the interaction between a protein (lysozyme) and mixed bilayers containing charged and neutral lipids should be useful to reveal mechanisms by which extrinsic proteins can affect the bilayer fluidity acting on the lipid lateral organization rather changing the strength of the lipid packing [16].

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Zusammenfassung – Vesikel bestehend aus geladenen (Phosphatidylsäure) und neutralen (Phosphatidylcholin) Lipiden wurden als Membranmodelle zur Untersuchung der lateralen Phasenseparation verwendet, die durch ein globuläres Protein, nämlich Lysozym, induziert wird. Die Fähigkeit des positiv geladenen Proteins, das auf der Vesikeloberfläche adsorbiert ist, Mikrodomänen unter Anreicherung der geladenen Lipidkomponente zu induzieren, wurde mittels kalorimetrischer Messungen (DSC) untersucht.

Die Proteinadsorption, die Denaturierungsrate und die Bildung von Mikrodomänen werden durch Variation des *pH* und der Salzkonzentration beeinflusst. Dies zeigt den starken Einfluss elektrischer Ladungen auf solche Phänomene. Einige der Resultate werden mit einem theoretischen, von den Autoren kürzlich entwickelten Modell interpretiert.