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SHIELDING OF PHOSPHOLIPID MONOLAYERS FROM PHOSPHOLIPASE C HYDROLYSIS BY α-LACTALBUMIN ADSORPTION

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SUMMARY

α-Lactalbumin interacts more strongly with lecithin and cardiolipin monolayers at pH 3∿4 than at pH 7 to 10. At physiological pH this protein does not penetrate monolayers of DPPC and cardiolipin above pressures of 30 dynes/cm. Enzymatic hydrolysis of these monolayers by phospholipase C (Clostridium Welchii) is inhibited partially or totally when α-lactalbumin is injected in the subphase prior to the enzyme injection.

INTRODUCTION

α-Lactalbumin is a protein that has no catalytic activity of his own but it causes the A protein present in the Golgi apparatus of mammary tissues to produce lactose (1, 2). Many studies were carried out on this protein in the last decade, mainly because of the extensive homology of its amino-acid sequence with egg-white lysozyme (3). Furthermore, both proteins have almost identical molecular weights, partial specific volumes, molecular conformation and similar optical rotary dispersion, circular dichroism and I.R. spectra (4, 5, 6, 7). However, there is a profound difference between the over-all charge of both proteins : α-lactalbumin has an isoionic point of about pH = 5, lysozyme of pH = 10.5. Data on conformational changes of both proteins in media of different pH exist (8, 9).

Numerous studies on the interaction of proteins with phospholipids in model systems have been published in recent years. The membrane proteins, according to Singer (1), can be divided in peripheral and integral proteins : he suggests that a peripheral protein (like α-lactalbumin or lysozyme) is attached to the exposed hydrophilic ends of an integral protein of the membrane and that the binding to the polar heads of lipids is weak.

The interaction between lysozyme and egg-lecithine and phosphatidylserine

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was studied in monolayers (10, 11), while Lenaz et al. studied the effect of binding of lysozyme to asolectin vesicles on phospholipase C hydrolysis (12). Similar information on α-lactalbumin is not available. In this paper we report the results of the monolayer studies of the adsorption of α-lactalbumin in different conditions. Information about the position of α-lactalbumin relative to the monolayer is obtained by measuring the surface pressure and by carrying out an enzymatic attack of the monolayer by phospholipase C when α-lactalbumin has been priorly injected into the subphase. We found that at pH 7,4 the adsorbed α-lactalbumin does not penetrate the monolayer, if the initial monolayer pressure is above 30 dynes/cm. It decreases the enzymatic hydrolysis of the lecithin monolayer by phospholipase C, either partially or totally, dependent on the initial pressure in the monolayer.

MATERIALS AND METHODS

Lipids : dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma. Purity and composition were confirmed by thin-layer and gas-liquid chromatography.
Cardiolipin from bovine heart is Sigma product C-3760. TLC shows this product to be pure, while GLC indicates that 90% of the fatty acids are unsaturated. L-α-phosphatidylserine (PS) from bovine brain is Sigma product P-6641. The purity is more than 95%. TLC shows that PA and PE are present.

Proteins : α-lactalbumin from Bovine milk was obtained from Sigma. Its purity was about 90%. It was further purified in a Sephadex G-100 column. Radioactive α-lactalbumin was prepared by iodonating the protein according to the method of Hunter and Greenwood (13). Four ^{125}I-isotopes are present per 100 protein molecules. Protein concentrations were determined spectrophotometrically with a Beckman D spectrophotometer, at 280 nm, making use of value $E^{1\%} = 20.1$ for α-lactalbumin (14).
Phospholipase C (Clostridium Welchii) was purchased from Sigma (product P-0264), enzymatic activity 250-300 I.U./mg protein.

Monolayer techniques :
The monolayers were obtained by spreading a chloroform solution of the phospholipids on a freshly cleaned surface of an aqueous solution containing the appropriate buffer. Different initial surface pressures are realized by spreading increasing amounts of phospholipids on the surface. The pressure changes at the air-water interface were determined in a teflon trough (2 cm deep and 54 cm^2 surface) with a Wilhelmy plate connected to a Cahn electrobalance. The enzymatic experiments were carried out in the same trough. The enzymatic action of phospholipase C was followed by the change of surface pressure π that occurs simultaneously. The α-lactalbumin was injected in aliquots up to 100 μl from a solution of 1 mg/ml. The amount of radioactive

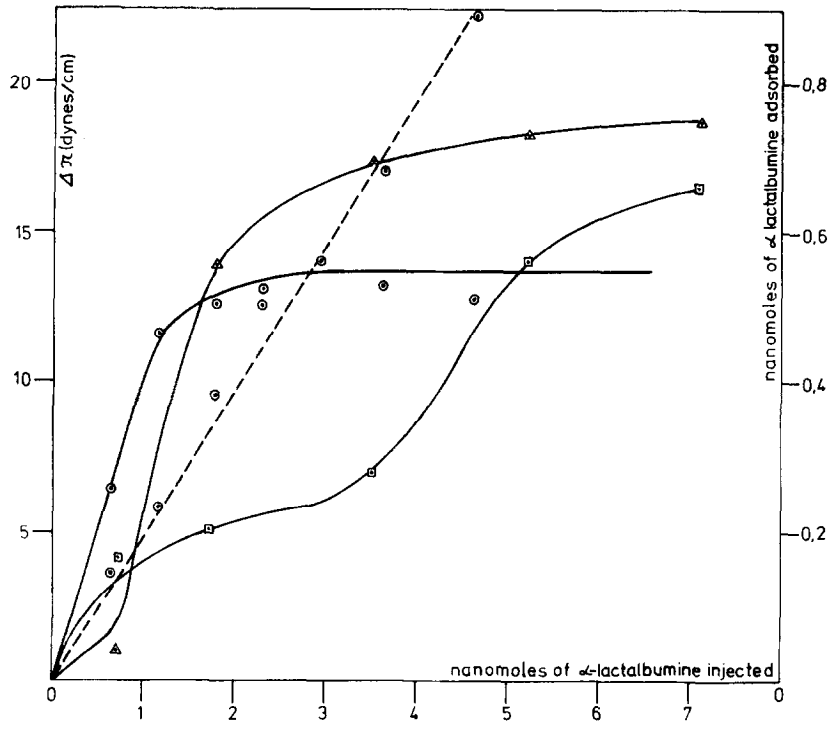

Figure 1. Increase of surface pressure of monolayers when different amounts of α-lactalbumin are injected in the subphase below monolayers of ◉ DPPC, ▲ phosphatidylserine (PS), ⊡ Cardiolipin. The initial surface pressure was 10 dynes/cm. The solution was at pH = 4.6 by acetate buffer 0.01 M. Temperature 27°5C. ⊙--- The amount of radioactive α-lactalbumin adsorbed to a DPPC-monolayer.

α-lactalbumin was measured by taking 2 x 0.5 ml solution from the subphase at the end of the experiments. The γ-radiation was measured with a Packard auto-gamma scintillation spectrometer.

RESULTS AND DISCUSSION

The increase in surface pressure after injection of different amounts of α-lactalbumin below monolayers of DPPC, PS and cardiolipin is plotted in figure 1 as a function of the number of moles injected. The initial pressure was set at 10 dynes/cm. Since α-lactalbumin interacts more strongly at acid pH, pH = 4.6 was selected to demonstrate the interaction. It is found, for DPPC and PS monolayers that for an injection of 3 nmoles of α-lactalbumin the maximum change of surface pressure is obtained, while for cardiolipin this value is $\pm$ 7 nanomoles. The same result is also obtained at other pH values (results not shown). The strength of the interaction at high adsorption of α-lactalbumin follows the sequence PS> Cardiolipin > DPPC. In the same

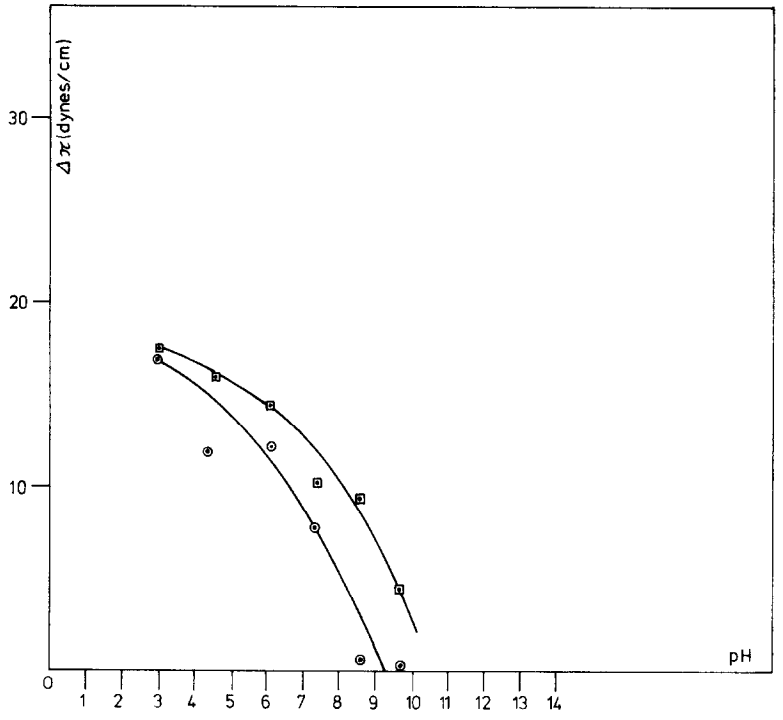

Figure 2. The increase in surface pressure $\Delta\pi$ at π_{in} = 10 dynes/cm when 6.8 nmoles of α-lactalbumin is injected in 50 ml subphase at different pH-values of the subphase. 3-6 acetate buffer, 7-10 TRIS-HCl buffer 0.01 M. Temperature = 27°5C. ⊙ Monolayer of DPPC. ⊡ Monolayer of Cardiolipin.

figure, by adsorption of R.A. α-lactalbumin we find that, although no further increase in surface pressure π is observed upon injection of more than 3 nmoles of protein, the adsorption of protein to the surface is still in progress. In agreement with Quinn and Dawson (15, 16), this can be explained by the fact that in the first part of the curve adsorption with penetration of the protein in the monolayer occurs while in the second part only adsorption but no penetration takes place. The acidic α-lactalbumin interacts strongly with DPPC and cardiolipin at low pH-values and weakly at high pH (see fig. 2), while the alkaline lysozyme, as found by Colacicco (10), interacts weakly with egg lecithin at low pH and strongly at high pH. Furthermore, the maximum initial pressure π at which α-lactalbumin still can penetrate is strongly decreased as the pH increases as shown in figure 3 : at pH 4.6 α-lactalbumin can penetrate a DPPC monolayer up to $\pm$ 33 dynes/cm, a cardiolipin monolayer up to 38 dynes/cm. At pH = 7.4 the limiting pressures are respectively 28 and 16. This behavior of both proteins cannot be explained only by taking into account the electrostatic interactions as both proteins are positively charged below

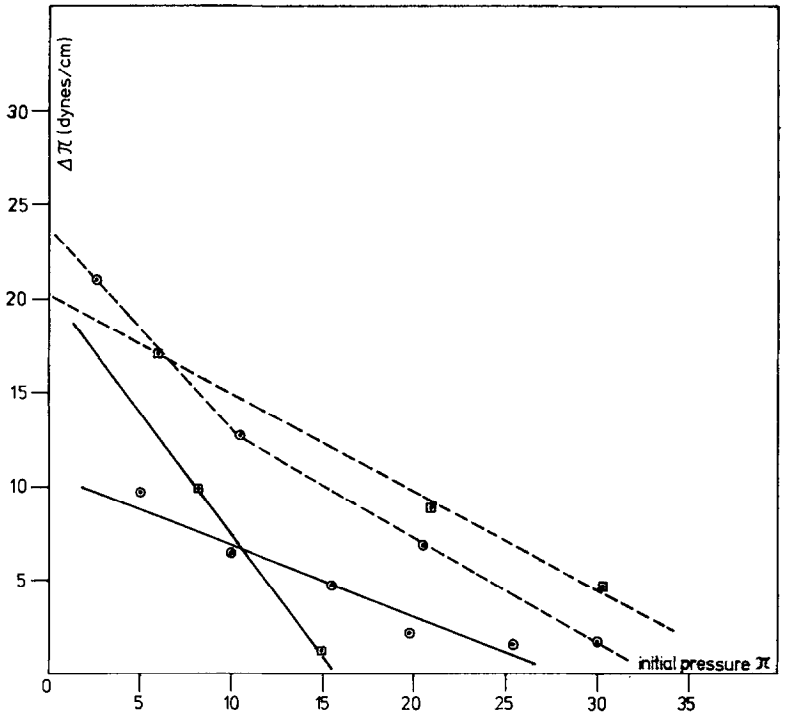

Figure 3. The increase in surface pressure π when 6.8 nmoles of α-lactalbumin at 27°C is injected as a function of the initial pressure π : ⊙ DPPC ⊡ cardiolipin. At pH = 7.4 —— , at pH = 4.6 ----.

pH = 5, while above 5 the netto-charge on α-lactalbumin becomes increasingly more negative, while lysozyme is still positive up to pH = +10. The maximum penetration around pH = 9 for lysozyme was attributed by Colacicco to the expansion of the protein in that pH region. The results of Warme et al. (17), namely that for α-lactalbumin at pH = 4.7 twice as much side-chains carboxyls are exposed to the surrounding than at pH = 7 , as well as the work of Kuwajima et al. (8) who found that a molecule α-lactalbumin at pH 3∿4 does not denaturate but rather expands about 30%, are indications that more hydrophobic regions of the molecule are exposed. The more hydrophobic character of the molecule facilitates not only the rate of adsorption to the surface (the rate is 4 times faster at pH = 3 than at pH = 10), but also its capacity to penetrate the monolayer. This hydrophobic penetration in the phospholipid monolayer does not exclude electrostatic interaction of other (charged) parts of the protein with the charged phosphatide ends of the monolayer.

Information about the peripheral protection of the phospholipid monolayer by a protein can be obtained by injection of an enzyme attacking the

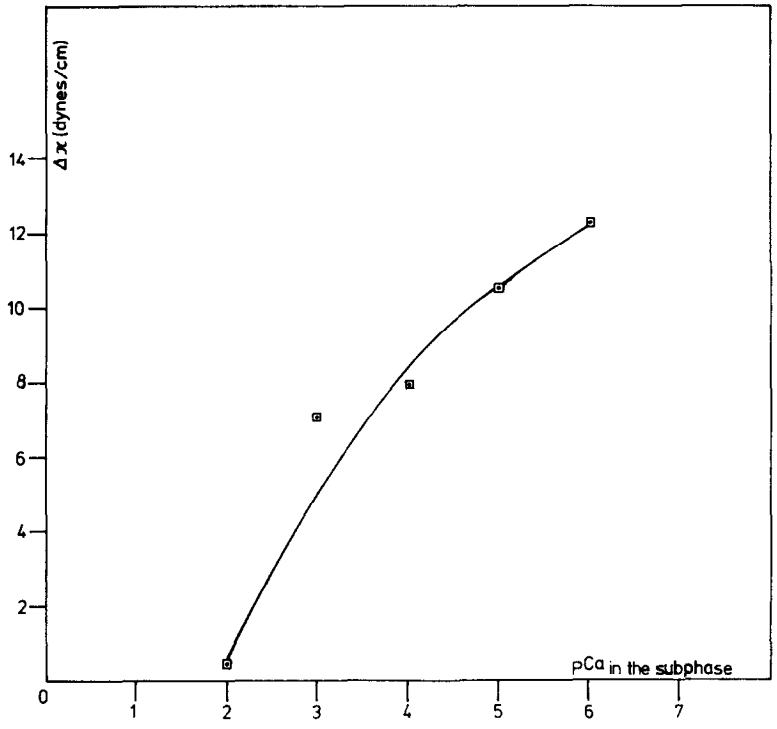

Figure 4. The increase in surface pressure when 5.2 nmoles of α-lactalbumin is injected under a monolayer of DPPC at π_{in} = 10 dynes with different $|Ca^{++}|$-concentrations in the subphase prior to protein injection pH = 4,6 acetate buffer 0.01 M. Temperature 27°5 C.

phospholipid in the monolayer. Phospholipase C (Clostridium Welchii) was therefore injected in the subphase after adsorption of α-lactalbumin had taken place. Since the subphase must contain $|Ca^{++}|$ = 0.01 M as a cofactor for the enzyme, the injection of Ca^{++} was done after the adsorption of α-lactalbumin reached equilibrium. If however Ca^{++} ions are injected prior to the protein, the penetration of the protein is inhibited. This is shown in figure 4. The change in surface pressure Δπ due to α-lactalbumin interaction becomes vanishingly small with increasing concentrations of Ca^{++} in the subphase. At $|Ca^{++}|$ = 0.01 M Δπ is negligible. No Δπ was measured when Ca^{++} was injected after the adsorption of α-lactalbumin reached equilibrium, Δπ was maintained on its prior value indicating that no α-lactalbumin was displaced.

The action of PL-ase towards a monolayer of DPPC with or without prior injection of α-lactalbumin is plotted in figure 5. Phospholipase C degrades DPPC monolayers up to 40 dynes/cm if no protein is present. This is in agreement with the systematic enzyme studies by Van Deenen et alii (18). In a second series of experiments 5.20 nmoles of α-lactalbumin was injected

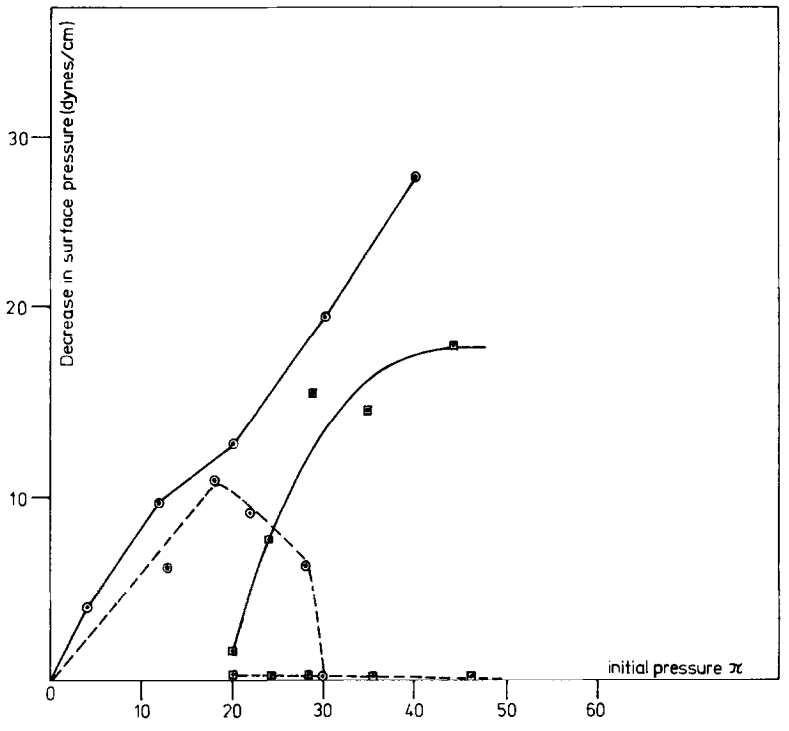

Figure 5. The decrease of surface pressure π by the action of phospholipase C (Clostridium Welchii) on a monolayer of DPPC or cardiolipin at different initial surface pressures π : pH = 7.4, $|Ca^{++}|$ = 0.01 M, temperature 27°C, phospholipase C = 0.5 mg/lit.
⊙ DPPC monolayer ⊡ Cardiolipin monolayer.
—— without α-lactalbumin in the subphase
---- when 6.8 nanomoles of α-lactalbumin are injected prior to phospholipase injection.

in the subphase. After equilibrium for the interaction protein-PL, the subphase was made 0.01 M in Ca^{++} and PL-ase C was injected. The initial pressure for these experiments is the pressure in the monolayer after the adsorption of the protein has reached equilibrium. It is observed that up to a pressure of 20 dynes/cm in the monolayer, the action of PL-ase C is comparable with or without α-lactalbumin present. Above 20 dynes/cm the action of PL-ase C decreases very fast. At 30 dynes/cm no surface pressure change due to enzymatic action is observed. This result agrees with a mechanism of adsorption in which at low $\pi_{initial}$ most injected protein penetrates the monolayer and does not shield the monolayer from the enzyme. Above π_{in} = 20 dynes/cm less protein is able to penetrate the monolayer, but most of it is still adsorbed to the phospholipid by ionic attraction to the polar heads of the lipids shielding completely the phospholipid from action of enzyme of the subphase.

Comparable results were obtained with cardiolipin monolayers.
In figure 5, the action of phospholipase C on monolayers of cardiolipin is shown as a function of the initial pressure. Below π_{in} = 20 dynes/cm no action of phospholipase on the pure monolayer is observed. The enzymatic activity increases up to π_{in} = 40 dynes/cm.
From figure 3 it is known that at pH = 7.4 α-lactalbumin cannot penetrate cardiolipin monolayers above π_{in} = 20 dynes/cm. If 5.2 nmoles of α-lactalbumin is injected prior to injection of the enzyme below a cardiolipin monolayer for which π_{in} > 20 dynes/cm no changes in surface pressure are observed, indicating that the α-lactalbumin does not penetrate the monolayer but is adsorbed to it and shields the phospholipid from the enzyme. An identical effect has been found for lysozyme by Lenaz et al. (12). The shielding by the peripheral protein is completely annealed if the solution is made 0,01 M in Ca^{++} prior to the protein injection. In that case the enzymatic action of phospholipase is the same with or without α-lactalbumin in the solution. This demonstrates that the protein is neither penetrating in, nor adsorbing to the monolayer in the presence of this bivalent cation.

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BIBLIOGRAPHY

1. Singer S.J., (1974) Ann. Rev. Biochem. 43, 805-833.
2. Brew K., Vanaman T.C. and Hill R.L., (1968) Biochemistry 59, 491-497.
3. Brew K., Vanaman T.C. and Hill R.L., (1967) J. Biol. Chem. 242, 3747-3749.
4. Kronman M.J., (1968) Biochem. Biophys. Res. Commun. 33, 535-541.
5. Tanaka F., Forster L.S., Pal P.K. and Rupley J.A. (1975) Biol. Chem. 250, 6977-6981.
6. Cowburn D.A., Brew K. and Gratzer W.B., (1972) Biochemistry 11, 1228-1234.
7. Barel A.O., Prieels J.P., Maes E., Looze Y. and Léonis J., (1972) Biochem. Biophys. Acta 257, 288-296.
8. Kuwajima K., Nitta K. and Sugai S., (1975) J. Biochem. 78, 205-211.

9. Kronman M.J., Jeroszko L. and Sage L.G., (1972) Biochem. Biophys. Acta 285, 145-166.

10. Colacicco G., (1973) Ann. N.Y. Acad. Sci. 195, 224-261.

11. Kimelberg H.K. and Papahadjopoulos D.,(1971)Biochem. Biophys. Acta 233, 805-809.

12. Lenaz G., Pasquali P., Bertoli E., Sechi A.M., Parenti-Castelli G. and Masotti L., (1972) Biochem. Biophys. Res. Commun. 49, 278-282.

13. Hunter W.R. and Greenwood F., (1962) Nature 194, 495-496.

14. Kronman M.J. and Andreotti R.E., (1964) Biochemistry 3, 1145-1151.

15. Quinn P.J. and Dawson R.M.C., (1969) Biochem. J. 115, 65-75.

16. Quinn P.J. and Dawson R.M.C., (1970) Biochem. J. 116, 671-680.

17. Warme P.K., Momany F.A., Rumball S.V., Tuttle R.W. and Scheraga H.A., (1974) Biochemistry 13, 768-782.

18. Demel R.A., Geurts Van Kessel W.S.M., Zwaal R.F.A., Roelofsen B. and Van Deenen L.L.M., (1975) Biochem. Biophys. Acta 406, 97-107.