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Résumé - Des couches monomoléculaires formées d'un mélange de phosphatidylserine et phosphatidylcholine sont déposées sur des plaques de chrome. L'association de prothrombine avec ces phospholipides est mesurée dans un tampon de soluté physiologique. Les résultats indiquent l'existence des deux complexes de prothrombine/phospholipides différents. La vitesse d'adsorption est contrôlée par la diffusion, avec une couche d'eau immobilisée à la surface de 20 µm d'épaisseur.

Abstract - Phospholipid monolayers consisting of mixtures of phosphatidylserine and phosphatidylcholine were deposited on chromium slides. Binding of prothrombin to these layers was measured in buffered physiological saline. Evidence is obtained for the existence of two different phospholipid/prothrombin complexes. The adsorption velocity is diffusion-controled with an apparent "unstirred layer" at the surface of 20 µm thickness.

Binding of proteins to phospholipid membranes plays an essential role in blood coagulation. An example is the calcium-dependent binding of prothrombin (factor II) to phospholipid which, in the presence of activated blood coagulation factors X and V, results in enzymatic cleavage of the prothrombin molecule and production of thrombin (factor II_a). Further understanding of this activation mechanism requires detailed knowledge of the binding constants involved and these have been measured in the last few years by a variety of experimental techniques (1, 2, 3, 4, 5). However, different techniques have produced values differing by a factor of 100. The present study may explain some of these discrepancies.

Materials and methods

Bovine prothrombin was prepared as described (6) and prothrombin concentrations were determined spectrophotometrically, using an extinction coeffient $E_{280}(1\%) = 14.4$ (7). Dioleoyl-phosphatidylcholine (DOPC) was purchased from Sigma Chemical Co and dioleoyl phosphatidylserine (DOPS) was prepared from DOPC by enzymatic conversion (8). Phospholipid concentrations were determined by phosphate analysis (9). Experiments were performed in 0.05 M trishydroxymethylaminomethane (Tris)-HCl buffer, pH= 7.0, containing 0.1 M NaCl and 0.01 M $CaCl_2$. Monolayers were prepared by the Langmuir-Blodgett techniques as described (5).
The chromium slide is placed in a quartz cuvette filled with buffer and a laser-produced beam of monochromatic light (λ = 632.8 nm), is reflected against the slide at an angle of incidence of 68 degrees. Before reaching the cuvette, the light passes through a polarizing prism, the "polarizer" P, and a quarter-wavelength plate, the "compen-

sator" C. After reflection, the light passes through a second polarizing prism, the "analyzer" A, and is detected by a photodiode. P and A are automatically rotated such that the resulting light intensity reaching the photodiode is kept minimal. From the positions of P and A thus obtained, the optical constants of the reflecting chromium surface can be determined. Knowing these constants, the refractive index n and the thickness d of a phospholipid or protein film, adsorbed to the slide, can be calculated from the changes in the positions of P and A due to the adsorption. The adsorbed mass Γ ($\mu g/cm^2$) of such a film can be obtained from the equation:

$$\Gamma = 0.3d(n^2-n_b^2)/[(n^2+2)(r(n_b^2+2)-v(n_b^2-1))] \quad (1)$$

where r and v are the specific refractivity and the partial specific volume of the adsorbed substance, both expressed in ml/g; n_b is the refractive index of the buffer solution and the thickness d is expressed in nm. Equation (1) is valid if the adsorbed layer consists of a mixture of buffer and phospholipid (or protein). If the refractive index n exceeds a value of n=1.6, equation (1) must be replaced by the equation for a pure substance:

$$\Gamma = 0.1d(n^2-1)/[r(n^2+2)] \quad (2)$$

Values used in the calculations were (5,10): n_b = 1.335; r = 0.274 ml/g and v = 0.889 ml/g for phospholipids; r = 0.236 ml/g and v = 0.710 ml/g for prothrombin. Detailed description of this ellipsometer (11) and the validation of equations (1) and (2) were published (5). Experiments were performed on slides with a surface area of 3.6 cm^2, placed in the cuvette filled with 4 ml of buffer. The buffer was continuously stirred, using a rotating magnetic stir, and kept at 37 $\pm$ 1°C by means of a Peltier element. Prothrombin, incubated in the same buffer, was added and the adsorption was followed by measuring the new positions of P and A every 3-5 seconds. When equilibrium was attained (10-60 min) a subsequent dose of prothrombin was added.

Results

Fig. 1 shows the adsorption of prothrombin on a monolayer containing 80% DOPS/20% DOPC. By measuring the equilibrium values of Γ as a function of the prothrombin concentration C in the buffer, the association constant K_a can be obtained. This is shown in Fig. 2. From these data it was calculated that for prothrombin concentrations up to 10 µg/ml the adsorption process on 100% DOPS can be described by a single class of binding sites with K_a = (2.01 $\pm$ 0.023) x 10^8 M^{-1} (mean $\pm$ SD) and Γ_{max} = 0.351 $\pm$ 0.005 $\mu g/cm^2$. For high prothrombin concentrations an extra amount of prothrombin is adsorbed. If the percentage of DOPC in the monolayer is increased, this biphasic behaviour becomes initially more pronounced with decreasing values of Γ_{max}. The adsoprtion on 20% DOPS/80% DOPC monolayers is still somewhat biphasic but can globally be described with values of K_a = (1.11 $\pm$ 0.34) x10^7 M^{-1} and Γ_{max} = 0.25 $\pm$ 0.02 $\mu g/cm^2$.
Measurement of lightscattering after rapid (stopped-flow) mixing of prothrombin with a vesicle suspension of phospholipids (12) has shown that the association process is very rapid with a $t_{\frac{1}{2}}$ of approximately 50 ms. The vesicles used in these experiments were sufficiently small ($\pm$ 25 nm diameter) to avoid limitation of the association velocity by diffusion. Comparing these data with the slow adsorptions as shown in Fig. 1, it is apparent that we have a completely diffusion-controled

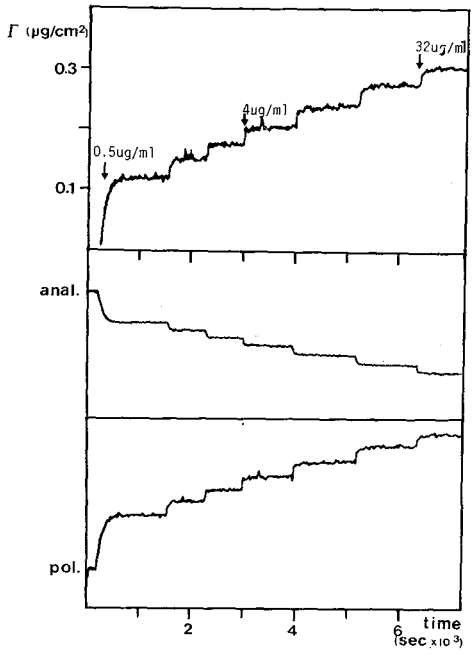

Fig.1. Stepwise adsorption of prothrombin on 80%DOPS/20%DOPC.

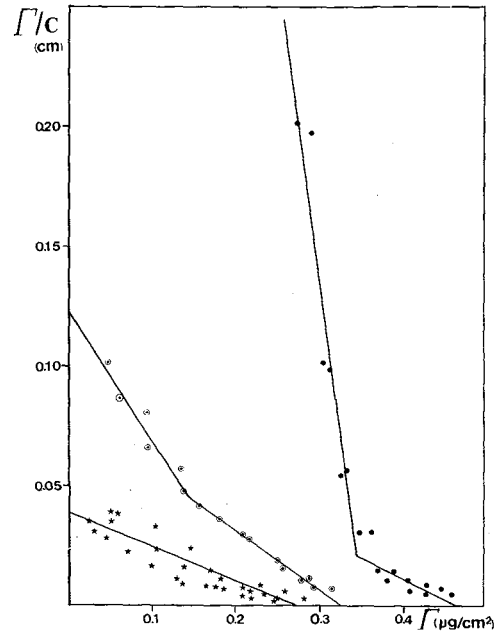

Fig.2. Scatchard plot for the adsorption of prothrombin on DOPS/DOPC mixtures. ●=100%DOPS;O=40%DOPS;★=20%DOPS.

situation. In that case one has in first approximation:

$$\frac{d\Gamma}{dt} = D(C - C_s)/\delta \qquad (3)$$

where D is the diffusion constant of prothrombin and C_s is the prothrombin concentration directly adjacent to the surface, which is separated from the bulk solution by an unstirred layer of buffer of thickness δ. In view of the rapid local association, C_s will be in equilibrium with Γ and equation (3) can be written as:

$$\frac{d\Gamma}{dt} = \frac{D}{\delta}\left[C - \frac{\Gamma}{K_a(\Gamma_{max} - \Gamma)}\right] \qquad (4)$$

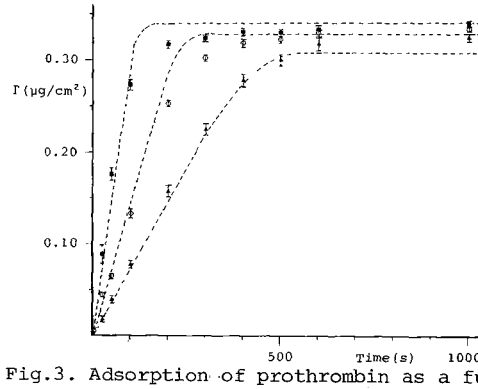

Fig.3. Adsorption of prothrombin as a function of time. C= 10 µg/ml (■),C= 5 µg/ml (O) and C= 2.5 µg/ml (▲). Mean ± SE, n=6. Broken lines present theoritical values.

Fig. 3 shows the adsorption velocity as a function of the prothrombin concentration C on a 100% DOPS monolayer. Taking the values K_a = 2.01 x 10^8 M^{-1} and Γ_{max} = 0.351 µg/cm^2 it is found that the solution of equation (4) may fit these data reasonably well with a value of D/δ = 2.4 x 10^{-4} cm/s. For the reported value of D for prothrombin of 4.8 x 10^{-7} cm^2/s (13) this implies that δ = 20 µm.

Discussion

The refractive index n and thickness d of a film deposited on a reflecting surface can be measured ellipsometrically even below monolay-

er coverage. It was shown, for instance, that correct values of n and d are obtained for fatty acids spread on a mercury surface in the range of 5% to 100% of full monolayer coverage (14). As a rule, however, values of n and d determined for films of less than 10 nm thickness have considerable experimental scatter. This scatter is not of a random nature but over- or underestimates of n correspond respectively to under- or overestimates of d. More specifically, the quantity $d(n^2-n_b^2)/(n^2+2)$ can be determined accurately in spite of fluctuating values of n and d. This explains why values of Γ as calculated from equation (2) contain a relatively small error, even for values of d below 5 nm (5).

Values of K_a for prothrombin-phospholipid complexes as reported in the literature show large variation. Using light-scattering and gel filtration techniques, both with suspensions of vesicles containing ± 25% phosphatidylserine (PS) and ± 75% phosphatidylcholine (PC), values of $K_a = 3 \times 10^6\ M^{-1}$ were obtained (1,2). Adsorption of tritium-labeled prothrombin on monolayers of 100% PS, spread on an aqueous subphase, resulted in a value of $K_a = 1.2 \times 10^8\ M^{-1}$ (3). These differences can be explained from the data shown in Fig. 2. Vesicle suspensions containing more than 30-50% of PS cannot be used in these binding experiments because of vesicle fusion at calcium concentrations in the millimolar range. On the other hand, prothrombin concentrations exceeding 10 µg/ml tend to solubilize phospholipid monolayers at the air-water interface, also interfering with such binding experiments.

The data presented in this study suggest the existence of a high-affinity prothrombin-DOPS complex for monolayers of pure DOPS. Addition of DOPC disturbs this complex and introduces a new phospholipid configuration with much lower binding affinity. A similar situation exists with respect to vesicle fusion. Vesicles of pure PS may aggregate by forming high-affinity anhydrous complexes. Addition of PC abolishes this phenomenon and results in water-containing complexes of much lower affinity (15).

The value of 20 µm found for the thickness of the unstirred layer is in reasonable agreement with a value of 30 µm found in an ellipsometric study on the adsorption of chymotrypsin on chromium slides coated with denatured albumin (16). The systematic differences between experimental and theoretical curves in Fig. 3 may be caused by the linear approximation (3).

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