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Interaction of Prothrombin and Its Fragments with Monolayers Containing Phosphatidylserine. 1. Binding of Prothrombin and Its Fragment I to Phosphatidylserine-Containing Monolayers†

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ABSTRACT: The adsorption isotherms of prothrombin and its fragment I on phosphatidylserine monolayers and on mixed monolayers of phosphatidylcholine and phosphatidylserine were determined by measuring surface radioactivity emanating from the tritium-labeled adsorbed proteins at 0.1 N NaCl and between 0 and 10 mM Ca^{2+}. The proteins were adsorbed from very dilute solutions, about 10 times more than in previous investigations on bilayer vesicles. The binding constants as obtained from the Scatchard plots were between 3×10^6 and 3×10^8 mol/L, depending on the experimental conditions. These values are between 2 and 50 times larger, respectively, than the binding constants obtained on bilayer vesicles. Prothrombin adsorbs appreciably also in the absence of Ca^{2+}. The significance of these results is discussed.

The importance of the vitamin K dependent γ-carboxyglutamic acid in the binding of prothrombin and factor Xa to phosphatidylserine-containing biological membranes and the blood clotting reactions has been well established (Nelsestuen, 1978; Esmon et al., 1975). Ca^{2+} is essential for the binding of these proteins to the negatively charged catalytic lipid surface, even though it can be to some extent replaced by other divalent ions (Nelsestuen & Lim, 1977; Nelsestuen et al., 1976; Furie et al., 1976).

The equilibria involved in the binding reaction are considered to be as shown in Scheme I (Nelsestuen & Lim, 1977), where Pt is the protein, Pt* is the protein after Ca^{2+}-induced conformational change, PL is the phospholipid, and i, j, and m are the molecularities of Ca^{2+} in each reaction. Most of the binding studies have been carried out on liposomes or bilayer vesicles. Quasielastic light scattering (Nelsestuen & Lim, 1977) and fluorescence quenching (Nelsestuen et al., 1976; Prendergast & Mann, 1977) were among the methods used for the investigation of the conformational relation of these proteins to their interaction with Ca^{2+} and with phospholipids. There is evidence that Ca^{2+} and phospholipid induce a conformational change in fragment I, but hardly any in prothrombin (Nelsestuen, 1976).

In a previous publication (Lecompte & Miller, 1980), we reported on the interaction of prothrombin with phosphatidylserine monolayers. The stoichiometry of the interaction was inferred from the measured surface radioactivity of ^3H-labeled prothrombin and of ^{45}Ca, while structural parameters were inferred from the electrical capacitance of the monolayer.

Scheme I

$$\left.\begin{array}{l} Pt + i\,Ca \overset{1}{\rightleftarrows} PtCa_i \overset{2}{\rightleftarrows} Pt^*Ca_i \\ \qquad\qquad\qquad\qquad + \\ PL + j\,Ca \overset{3}{\rightleftarrows} PL\cdot Ca_j \end{array}\right\} + m\,Ca \overset{4}{\rightleftarrows} Pt^*PL\cdot Ca_{(i+l+m)}$$

In the first paper of this series we wish to describe the adsorption isotherms of prothrombin and of its fragment I on the monolayer of phosphatidylserine mixed with phosphatidylcholine at different ratios. The binding constants and stoichiometry, as well as the Ca^{2+} requirement as inferred from these measurements, will be compared with the results obtained with lipid dispersions.

Materials and Methods

The phospholipids (egg lecithin and ox brain phosphatidylserine, purchased from Lipid Products, Nutfield, England) were supplied in chloroform–methanol solution. For spreading, samples were evaporated in a stream of nitrogen, the lipid content was determined by weight, samples were dissolved in hexane, and the desired compositions were obtained by mixing the hexane solutions.

A two- to threefold excess of the lipid was spread over an aqueous phase containing 0.1 M NaCl and 10^{-3} M Tris at pH 7.8. The excess assured a fully compressed monolayer in equilibrium with the collapsed excess lipid layers.

Human prothrombin and its fragment I were purified by R. Benarous and J. Elion, according to Mann (1976).

Radioactive ^3H-labeled prothrombin and fragment I were prepared by oxidizing their sialic acid with sodium metaperiodate and then by reducing the obtained aldehyde with sodium [^3H]borohydride (Butkowski et al., 1974). The radioactive [^3H]borohydride was purchased from the Radiochemical Center, Amersham, England.

For determination of adsorption onto the lipid monolayer, between 10 and 100 μL of radioactive protein solution was injected into 15 mL of buffer solution underneath the spread

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monolayer, up to a final concentration between 0.5 and 10 μg/mL. To achieve effective stirring of the submonolayer solution, half of it was repetitively but carefully retrieved and reinjected with a syringe, without disrupting the monolayer. Surface radioactivity was then counted with an ultrathin (~200 nm), supported, end-window gas-flow counter as described elsewhere (Frommer & Miller, 1966). The counter was calibrated with a spread monolayer of [^{3}H]oleic acid. The surface concentration of the radioactive protein is given by

$$\Gamma_{P^+} = \frac{(\text{cpm})^g_p N_p (\text{cpm})^b_{OA}}{A(\text{cpm})^b_p (\text{cpm})^g_{OA}} \quad (1)$$

where $(\text{cpm})^g_{OA}$ and $(\text{cpm})^b_{OA}$ are the surface and the bulk (scintillation) counts per minute of an equal sample of oleic acid, $(\text{cpm})^g_p$ is the surface count of the protein from the total area A, and $(\text{cpm})^b_p$ is the scintillation count of N_p protein molecules.

Protein concentrations were determined by ultraviolet absorbance using an $\epsilon^{1\%}_{280\text{nm}} = 14$ for prothrombin and $\epsilon^{1\%}_{280\text{nm}} = 12$ for fragment I.

Results

The adsorption from very low concentrations is a very slow process, and equilibrium surface concentration could be reached only after a very long time, even when the adsorption itself is instantaneous and the process is completely diffusion controlled. Under the experimental conditions of gentle stirring underneath the monolayer, the diffusion was through a diffusion layer of constant thickness δ. Assuming that the monolayer composed of equivalent adsorption sites is in Langmuirian equilibrium with the adjacent layer in the bulk, negligibly affected by lateral infraction, the diffusion differential equation can be written

$$\frac{d\Gamma}{dt} = \frac{D}{\delta}\left(c - \frac{K_d\Gamma}{\Gamma_{max} - \Gamma}\right) \quad (2)$$

where Γ and Γ_{max} are the surface concentrations of the protein at time t and the maximal surface concentration, respectively, which can be reached by increasing the bulk concentration c under the given conditions, and K_d is the dissociation constant of the protein from the lipid layer. The solution of the differential equation is

$$\frac{D}{\delta}t = \frac{\Gamma}{c + K_d} - \frac{K_d\Gamma_{max}}{(c + K_d)^2}\log\frac{c\Gamma_{max} - (c + K_d)\Gamma}{c\Gamma_{max}} \quad (3)$$

At short time, Γ is proportional to t, and from the initial straight lines, D/δ can be evaluated. At longer times, the second term takes over completely and eq 2 becomes

$$\Gamma = \frac{c\Gamma_{max}}{c + K_d}\left[1 - \exp\left(-\frac{(c + K_d)^2 Dt}{K_d{}^2\delta}\right)\right] \quad (4)$$

At long enough time, the equilibrium surface concentration $\Gamma = c/(c + K_d)\Gamma_{max}$ is approached. For construction of our adsorption isotherms, we tried to approach these equilibrium surface concentrations. They were assumed to be reached, when doubling the time did not result in a significant change in surface radioactivity.

(1) Adsorption Isotherms of Prothrombin. (a) Adsorption onto Pure Phosphatidylserine Monolayer. In Figure 1a the surface concentrations of prothrombin on the compressed phosphatidylserine monolayer, as a function of its initial concentration, in the presence of different concentrations of Ca^{2+} are given. These are not true adsorption isotherms, since

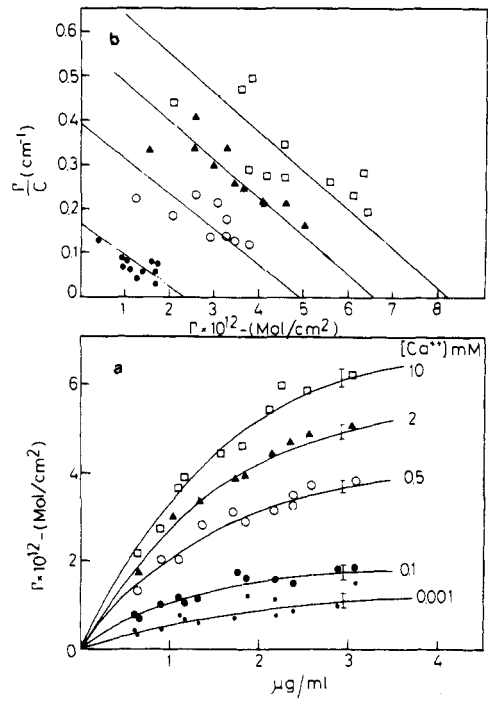


FIGURE 1: Adsorption of prothrombin on compressed phosphatidylserine monolayers. (a) Surface concentration of adsorbed prothrombin in mol/cm^2 as a function of its concentration in the subphase, right after its injection. (b) Scatchard plot. Ratio of the equilibrium values of prothrombin surface concentration Γ (mol/cm^2) to its free concentration [Pt] (mol/cm^3) against the surface concentration [Ca^{2+}] (mM): (□) 10; (▲) 2; (○) 0.5; (●) 0.1.

the bulk concentration is depleted during the course of adsorption. Between 10^{-1} and 10 mM Ca^{2+}, the adsorption isotherms (also the real ones) are strongly dependent on calcium concentration. Below 10^{-1} /mM Ca^{2+} and down to below 10^{-3} mM Ca^{2+} the adsorption remains almost constant and significant $\Gamma = 1.5 \times 10^{-12}$ mol/cm^2. It has been shown previously (Lecompte & Miller, 1980) that even at concentrations as low as 10^{-3} mM Ca^{2+} is coadsorbed with prothrombin. In the presence of 1 or 2 $\times 10^{-3}$ mM Ca^{2+} the surface concentration of Ca^{2+} is proportional to that of the adsorbed prothrombin, about 10 molecules of Ca^{2+} being coadsorbed with 1 molecule of prothrombin. However, even in the absence of Ca^{2+} or in the presence of EDTA, the prothrombin adsorption remains significant.

In Figure 1b the Scatchard plot (Γ/c) vs. Γ is presented for different Ca^{2+} concentrations, where c is the true prothrombin concentration in equilibrium with the surface. As can be seen from this figure, the slopes of the straight lines obtained at the different Ca^{2+} concentrations above 10^{-1} mM are about the same; only the intercepts differ. Thus, the binding constant K_A (or its reciprocal $-K_d$), given by the slope, is in this region independent of Ca^{2+} concentration ($K_A \simeq 1.2 \times 10^8$ L/mol); only the maximal surface concentration, Γ_{max}, or the number of binding sites obtained from the intercept is dependent. According to Scheme I, this would suggest that equilibria 1 and 2 involving prothrombin and Ca^{2+} are, at these Ca^{2+} concentrations, pushed completely toward the prothrombin–Ca complex. This is in accordance with the stoichiometric coadsorption of Ca^{2+} with prothrombin at 10^{-3} mM Ca^{2+} (Lecompte & Miller, 1980). However, this would contradict the finding that at 0.5 mM Ca^{2+} only half of the sites on prothrombin are occupied by Ca^{2+} (Nelsestuen, 1976). Therefore, another explanation is to be considered.

We may assume that the number of binding sites on the monolayer, given by $(\Gamma_{max} - \Gamma_0)$, where Γ_0 is the maximal

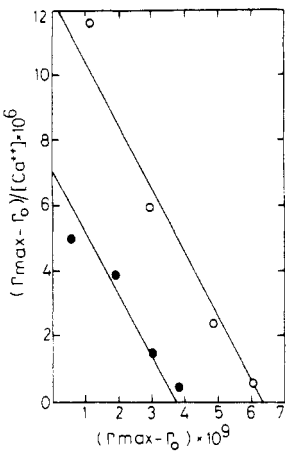


FIGURE 2: Plot of maximal surface concentration of prothrombin (●) and of fragment I (○) on condensed phosphatidylserine monolayer, in the presence of calcium at a concentration $[Ca^{2+}]$; $\Gamma_{max} - \Gamma_0$ (the maximal surface concentration in the absence of calcium) divided by $[Ca^{2+}]$ against $\Gamma_{max} - \Gamma_0$. The surface concentrations are given in mol of amino acid residues/cm^2.

protein surface concentration at $[Ca^{2+}] = 0$, is proportional to the number of Ca^{2+} bound to the monolayer according to equilibrium 3 in Scheme I. If this assumption is correct and the Ca^{2+} adsorption in the presence of excess monovalent salt (0.1 M NaCl) is of the Langmuirian type, the plot of $(\Gamma_{max} - \Gamma_0)/c_{Ca^{2+}}$ vs. $(\Gamma_{max} - \Gamma_0)$ should give a straight line. As seen from Figure 2, this is indeed the case. The slope of the line represents in this case the binding constant of Ca^{2+} to the phosphatidylserine monolayer, which is about 2×10^3 L/mol, in agreement with Nelsestuen & Lim (1977). This is, however, not the true association constant, since it includes the electrostatic term

$$K = K_{true} \exp(-2F\psi(0)/RT) \quad (5)$$

where $\psi(0)$ is the potential in the charged plane. K_{true}, as obtained by McLaughlin et al. (1971) and by Ohki & Sauve (1978), is 0.1 L/mol in the presence of low Ca^{2+} concentrations, but it increases to about 0.2 at 10 mM Ca^{2+}. The apparent binding constant should vary according to eq 5, with calculated values of $\psi(0)$ by the Grahame equation varying between about 3×10^3 for 0.1 mM Ca^{2+} and about 4×10^2 for 10 mM Ca^{2+}. The values inferred from Figure 2 are about 5 times higher. This may be due to some cooperative effect of enhancement of Ca^{2+} binding by the adsorbing prothrombin molecules.

(*b*) *Adsorption of Prothrombin onto Condensed Monolayers Containing 25% Phosphatidylserine and 75% Phosphatidylcholine.* Adsorption measurements of Pt onto monolayers containing 25% phosphatidylserine were carried out in the presence of 0.5 and 2 mM Ca^{2+}. The results are presented in Figure 3a, where in addition to the adsorption isotherms the Scatchard plots are also given. The respective Γ_{max} values at these two Ca^{2+} concentrations are 1×10^{-12} and 1.75×10^{-12} mol/cm^2, respectively, as compared with 4×10^{-12} and 6.2×10^{-12} mol/cm^2 adsorbed on pure phosphatidylserine monolayers. Thus, within experimental error, the maximal adsorption at any Ca^{2+} concentration is proportional to the negative charge of the monolayer. It follows from this that the dependence of the adsorption on the mixed monolayer on Ca^{2+} concentration is parallel to that on pure phosphatidylserine monolayer. This is demonstrated in Figure 2 where $\Gamma/[Ca^{2+}]$ is plotted against Γ.

The slopes of the Scatchard plots (Figure 3b) at the two concentrations of Ca^{2+} are about the same, corresponding to

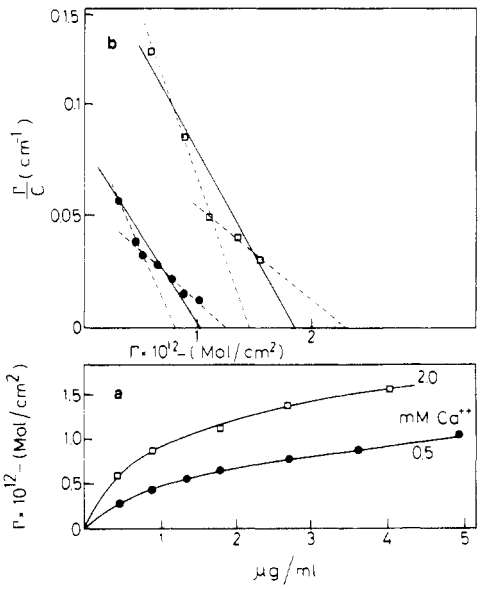


FIGURE 3: Adsorption of prothrombin on a compressed monolayer containing 75% phosphatidylcholine and 25% phosphatidylserine. (a) Surface concentration of adsorbed prothrombin as a function of its concentration in the subphase at time of its injection. (b) Scatchard plot $\Gamma/[Pt]$ against Γ.

binding constants between 0.8×10^8 and 1×10^8 L/mol. These values are still within the experimental error of the values $(1-1.3) \times 10^8$ L/mol obtained on pure phosphatidylserine monolayers.

All these experiments were done in the very low protein concentration region, since a large excess of prothrombin tends to solubilize the lipid layer; furthermore, we avoid the self-association of prothrombin or fragment 1, described by Prendergast & Mann (1977) and Jackson et al. (1979), which occurs at higher protein concentrations. It stands to reason, and this possibility is indicated by the results, that at higher concentrations further adsorption would proceed with a lower binding constant. Indeed, the binding constants obtained (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977) at considerably higher prothrombin concentrations (above 30 μg/mL) at a molar fraction of ~0.25 of phosphatidylserine in phosphatidylcholine were about 2 to 3×10^6 L/mol or about 30 to 50 times lower than those obtained by us. This difference can be attributed to the difference in the concentration region, but it also could be due to the fact that in the present case the adsorption was on a planar lipid monolayer, while in the other case it was on bilayer vesicles with a small radius of curvature. The packing of the head groups on the curved surface differs from that on the planar surface, which may influence the binding constants.

(*2*) *Adsorption of Fragment I.* The apparent adsorption isotherms of fragment I on a compressed phosphatidylserine monolayer, as obtained by measuring surface radioactivity after injecting fragment I underneath the monolayer to the indicated concentration, are shown in Figure 4a. Surface concentrations were calculated from the "constant" surface radioactivity reached after 15–80 min, depending on the bulk concentration. Unlike the case of prothrombin, very little adsorption is observed at 10^{-3} mM Ca^{2+}. The adsorption at the higher concentrations of Ca^{2+} is considerably higher than that of prothrombin, even if one takes into account the differences in molecular weight and one compares weights per unit area. For example, at 2 mM Ca^{2+} the maximal surface concentration of prothrombin is about 0.43 μg/cm^2, while that of fragment I is about 0.62 μg/cm^2.

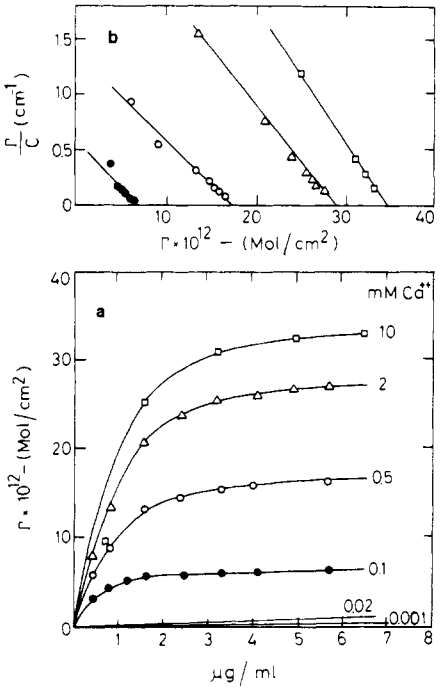
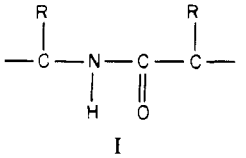


FIGURE 4: Adsorption of fragment I on compressed phosphatidylserine monolayers. (a) Surface concentration of adsorbed fragment I, Γ_{FI} (mol/cm^2), as a function of its concentration in the subphase right after injection. (b) Scatchard plot: Γ_{FI} divided by the equilibrium value of the free concentration of fragment I, C_{FI}, in mol/cm^3 against Γ.

In Figure 4b the Scatchard plots constructed from the adsorption isotherms are shown. Here, the bulk solutions were strongly depleted by adsorption. According to the slopes, the binding constants seem to increase slightly with the Ca^{2+} concentration, but by and large they do not differ appreciably from the binding constants of prothrombin, varying between 8×10^7 and 1.2×10^8 L/mol. These binding constants are between 50 and 80 times larger than those obtained on the bilayer vesicle composed of phosphatidylcholine and phosphatidylglycerol at a 1:1 ratio (Dombrose et al., 1979). Plotting $\Gamma_{max}/[Ca^{2+}]$ against Γ_{max} (Figure 2), one can obtain the maximal adsorption at higher concentrations of Ca^{2+}. It is about 6.3×10^{-9} mol of amino acid residues per cm^2, which is 3.6×10^{-11} mol/cm^2 of fragment I, or one molecule occupies an area of 460 Å^2. If the molecule is attached to the phosphatidylserine monolayer through 10 Ca^{2+} bridges by the γ-carboxyglutamic acid (Glu), an area of 46 Å^2 is allowed per Glu, the peptidic bond section (I) being 7.2 Å in length.



I

The adsorption decreases with the replacement of the negatively charged phosphatidylserine by the neutral phosphatidylcholine. Examples of adsorption isotherms on monolayers containing 50 and 25% phosphatidylserine are presented in Figure 5a. The Scatchard plots in Figure 5b indicate that, with decreasing negative charge of the monolayer, not only does the maximal adsorption decrease but also the binding constants are affected. In this respect, the behavior of fragment I is different from prothrombin, where the binding constants were only a little affected by diluting the phosphatidylserine in the surface. At 50% phosphatidylserine, the binding constant of fragment I is about 15 times smaller than

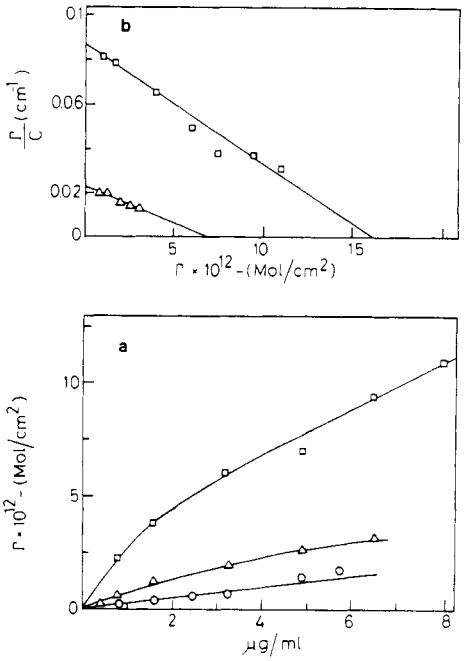


FIGURE 5: Adsorption of fragment I on compressed mixed lipid monolayers phosphatidylcholine + phosphatidylserine. (a) Γ_{FI} as a function of the subphase concentration at time of injection: (□) 50% phosphatidylserine and 2 mM Ca^{2+}; (△) 25% phosphatidylserine and 2 mM Ca^{2+}; (○) 25% phosphatidylserine and 0.1 mM Ca^{2+}. (b) Scatchard plot Γ_{FI}/C_{FI} against Γ_{FI}: (□) 50% phosphatidylserine and 2 mM Ca^{2+}; (△) 25% phosphatidylserine and 2 mM Ca^{2+}.

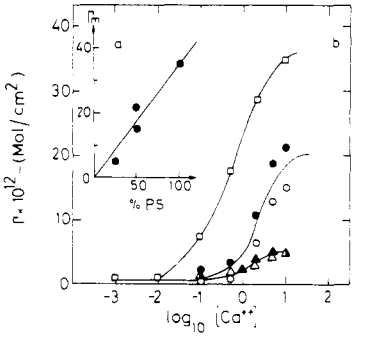


FIGURE 6: Dependence of maximal surface concentration of fragment I on log [Ca^{2+}] (mM). Inset: Maximal adsorption at high Ca^{2+} concentration as a function of monolayer composition. (□) 100% phosphatidylserine; (○,●) 50% phosphatidylserine; (△,▲) 25% phosphatidylserine.

at 100% phosphatidylserine and it reaches values only 3 to 5 times larger than those obtained on vesicles (Dombrose et al., 1979). Lowering the phosphatidylserine concentration in the monolayer to 25% causes a further twofold decrease in the binding constant.

The dependence on Ca^{2+} concentration of the maximal adsorption on monolayers containing different molar fractions of phosphatidylserine is presented in Figure 6. In the inset the maximal adsorption extrapolated from the Scatchard-type plot of $\Gamma_{max}/[Ca^{2+}]$ against Γ_{max} is presented as a function of the phosphatidylserine molar ratio in the phosphatidylserine–phosphatidylcholine monolayer. The maximal adsorption is proportional, within experimental error, to the phosphatidylserine content of the monolayer.

Discussion

The adsorption measurements on lipid monolayers reported here were carried out from protein solutions which were about one order of magnitude less concentrated than in the adsorption

Scheme II

$$Pt + PL \overset{K_a}{\rightleftharpoons} PL{\cdot}Pt \underset{K_b}{\overset{nCa}{\longrightarrow}} PL{\cdot}PtCa_n$$

experiments on bilayer vesicles reported previously (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977; Dombrose et al., 1979). This may explain some of the apparent discrepancies between the results obtained and the conclusions inferred. However, the main differences probably result from the differences in head group arrangement on the highly curved surface of a vesicle and on a planar monolayer.

There are a few findings in these experiments which call for further discussion. (1) Even below 10^{-3} mM Ca^{2+} and up to 1.5×10^{-12} mol/cm^2, prothrombin is adsorbed, and this maximal adsorption stays constant until close to 10^{-1} mM Ca^{2+}. Adsorption at these low Ca^{2+} concentrations was also obtained on phospholipid vesicles (Nelsestuen, 1976). Coadsorption of Ca^{2+} on phosphatidylserine monolayers from these low concentrations calls for special consideration. (2) In the entire region between 10^{-1} and 10 mM Ca^{2+} the binding constant on phosphatidylserine monolayers is practically independent of calcium concentration and stays for prothrombin and for fragment I around 10^8 L/mol. For prothrombin, this value decreases by less than 50% when phosphatidylserine in the monolayer is lowered to 25%, and it is at least 20 times larger than those reported on vesicles (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977). (3) The maximal surface concentration of prothrombin reached at any Ca^{2+} concentration between 10^{-1} and 10 mM seems to be proportional to Ca^{2+} adsorption on the lipid monolayer.

Scheme I is not sufficient to explain this behavior, particularly not the coadsorption of Ca^{2+} from concentrations in the 10^{-3} mM region. It has been shown that, at these low concentrations in the presence of 0.1 M NaCl, Ca^{2+} does not bind appreciably to prothrombin (Benson & Hanahan, 1975; Benarous et al., 1976; Nelsestuen, 1976) or to fragment I (Nelsestuen et al. 1976; Henriksen & Jackson, 1975; Prendergast & Mann, 1977). Thus, the prothrombin–phospholipid complex formed by penetration of the prothrombin into the lipid layer has a much higher affinity for Ca^{2+} than either pure prothrombin or pure phospholipid. At these low Ca^{2+} concentrations, there is a prothrombin–phospholipid complex but no fragment I–phospholipid complex. Penetration of prothrombin into phospholipid monolayers was demonstrated by surface electrochemical methods (Lecompte & Miller, 1980) and will be discussed in detail in the subsequent publications of this series. Penetration combined with adsorption reveals interaction sites in addition to fragment I, which allow the reaction shown in Scheme II to take place up to a certain surface coverage. The binding constant of Scheme II is nearly the same as that at the higher concentration of Ca^{2+}, $\sim 5 \times 10^7$ L/mol. Fragment I does not bind to the phosphatidylserine monolayer in the absence of Ca^{2+} from low bulk concentration, <10 μg/mL. Binding to phospholipid vesicles in the absence of Ca^{2+} was observed from higher bulk concentrations, >50 μg/mL (Nelsestuen, 1976). This is in accord with the cooperative penetration or perturbance of the lipid layer, which will be shown in subsequent papers.

Scheme I, which is supposed to represent prothrombin–phospholipid interaction, should apply in the millimolar region of Ca^{2+} concentration. It has to be modified in order to fit adsorption on an infinite plane. If the maximum number of binding sites on a phospholipid monolayer as $Ca \rightarrow \infty$ is $\Gamma_{max}(\Theta_{PL}^{Ca} = 1) = \Gamma^*_{max}$, then the actual number of binding sites at a particular concentration of Ca^{2+} will be $\Theta_{PL}^{Ca}\Gamma^*_{max}$:

$$\Theta_{PL}^{Ca} = \frac{K_3[Ca^{2+}]}{1 + K_3[Ca^{2+}]} \quad (6)$$

However, up to a certain surface coverage, when hydrophobic interactions contribute to the free-energy adsorption, Γ_{Pt} will be given according to Scheme II by

$$\Gamma_{Pt} = \frac{K_a[Pt]\Gamma^*_{max}}{1 + K_a[Pt]} \quad (7)$$

The surface complex creates strong binding sites for Ca^{2+}. Equations 6 and 7 imply negligible effect of lateral interaction on the adsorption isotherms. This assumption is in keeping with the resulting Scatchard plots.

The very weak dependence of K_4 on the concentration of Ca^{2+}, at the higher concentrations of Ca^{2+} when Scheme I should hold, means that it is practically the same for prothrombin molecules loaded to different degrees with Ca^{2+}. In the measured concentration regions of prothrombin, K_4 seems also to be only a little dependent on the phosphatidylserine content of the monolayer. The maximal surface concentration of prothrombin adsorbed on a pure phosphatidylserine monolayer at 2 mM Ca^{2+} is 6.2×10^{-12} mol/cm^2, corresponding to about 2700 Å^2/molecule. On a monolayer containing 25% phosphatidylserine, the limiting surface area is above 10000 Å^2 per molecule, which is about 4 times more, so that there is a tight correlation between the limiting area occupied by prothrombin molecules and phosphatidylserine concentration. At lower concentrations of phosphatidylserine in the monolayer, the maximal surface concentrations are proportionally lower. The area occupied by a prothrombin molecule at maximal hexagonal packing oriented with its long axis perpendicular to the surface is 1750 Å^2. These areas per molecule of adsorbed prothrombin have been approached when adsorbed on vesicles of 75% phosphatidylcholine and 25% phosphatidylserine from concentrations up to 100 μg/mL under otherwise similar conditions (Nelsestuen & Broderius, 1977). This explains at least in part the considerably higher binding constants obtained here in the very dilute solutions. The adsorption is probably biphasic, with a lower binding constant at the higher surface concentration. Examining the adsorption isotherms and their Scatchard plots (Figure 3b), we find an indication for this biphasic behavior. It is our impression that the smaller binding constants indicated for the higher prothrombin concentrations are still about 10 times higher than those obtained on vesicles. It seems, therefore, that the interrelation between the planarity of the surface affecting the head group orientation and pack and between prothrombin adsorption is of importance.

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Interaction of Prothrombin and Its Fragments with Monolayers Containing Phosphatidylserine. 2. Electrochemical Determination of Lipid Layer Perturbation by Interacting Prothrombin and Its Fragments†

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ABSTRACT: The effect of prothrombin, its fragments I and II, and thrombin on the capacitance of phosphatidylserine-containing monolayers transferred on a mercury electrode has been measured. An increase in capacitance from 1.5 to 7 $\mu F/cm^2$ and the appearance of a pseudocapacitance peak due to cystine–cysteine redox reaction on the electrode indicate perturbation of the lipid layer structure and its penetration by the protein. We concluded from the pseudocapacitance peaks that only 0.1 or less of the cystine residues on the adsorbed prothrombin molecules can penetrate the lipid layer to react on the electrode. The penetration occurs at the air–water interface, and the increased capacitance values are observed immediately upon replacing the air by the mercury surface. The pseudocapacitance peak, on the other hand, evolves slowly after contacting the monolayer by the positively charged mercury electrode which adsorbs the cystine residues. The kinetics of the evolution of the pseudocapacitance peak and its dependence on the frequency of the ac modulation are related to the dynamic properties of the protein interacting with the lipid surface. It shows that the Ca^{2+}-mediated binding is more pliable at lower negative charges on the lipid layer, which may affect its function in the prothrombin–thrombin transformation. The penetration of the three fragments depends on the monolayer composition, but only fragment I resembles prothrombin in its effect, depending also on Ca^{2+} concentration. Fragment II does not penetrate a mixed monolayer of 75% phosphatidylcholine and 25% phosphatidylserine, while the positively charged and more hydrophobic thrombin does. The significance of the results with respect to the possible role of the different fragments in the prothrombin–phospholipid interaction is discussed.

In the preceding paper (Lecompte & Miller, 1980), we determined the adsorption isotherm of prothrombin on phosphatidylserine-containing fully compressed monolayers from 0.1 M NaCl solution in the presence of different concentrations of Ca^{2+} up to 10 mM. Ca^{2+} enhances adsorption by bridging the negative charges of phosphatidylserine in the monolayer with those of the γ-carboxyglutamic acid residues of fragment I or of prothrombin. However, appreciable adsorption was obtained also in the absence of Ca^{2+}, which indicates that other interactions besides the electrostatic ones have to take place. These other interactions, either alone or in combination with the electrostatic ones, are expected to perturb the continuous structure of the lipid monolayer. When a spread lipid monolayer is brought in contact with an electrode from the gaseous phase, its impedance and other electrical properties can be investigated (Pagano & Miller, 1973; Miller et al., 1976; Miller & Rishpon, 1977). The capacitance of an intact lipid monolayer is characteristic of a hydrocarbon layer, one hydrocarbon chain length thick, varying between 1.2 and 1.7 $\mu F/cm^2$. If the continuity of the monolayer is perturbed or penetrated by an interacting molecule of higher polarity, an increase in capacitance proportional to the degree of perturbance or penetration is observed. In the case when the penetrating molecule contains electroactive groups undergoing electrode reaction, a pseudocapacitance peak is obtained that is proportional in size to the access of these groups through the lipid layer to the electrode surface. In the case of prothrombin and of many other proteins, cystine may serve as such an electroactive group. Cystine is strongly adsorbed on the mercury surface at positive potentials of the redox potential forming a charge-transfer complex (Kolthoff & Barnum, 1941). The surface complex is then reduced at the redox potential giving rise to the pseudocapacitance peak. The total surface reaction disregarding the stoichiometric relations is

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