Adsorption of Bovine Prothrombin to Spread Phospholipid Monolayers

Eric H. Ellison and Francis J. Castellino
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556 USA

ABSTRACT The interaction of bovine prothrombin with phospholipids was measured, using as the lipid source monolayers spread at the air-buffer interface. Fluorescence spectroscopy was implemented to determine the equilibrium concentration of free prothrombin in the aqueous subphase of the protein-monolayer suspensions, in a continuous assay system. The increase in surface pressure (π) from the protein-monolayer adsorption was also measured and, with values of the adsorbed protein concentration (c_s), was used to calculate $dc_s/d\pi$. At a particular phosphatidylserine (PS) content of liquid-expanded (LE) phosphatidylcholine (PC)/PS monolayers, $dc_s/d\pi$ was independent of the initial surface pressure (π_i), when this latter value exceeded 30 mN/m. However, $dc_s/d\pi$ varied significantly with the relative PS content of the monolayer. Values of the equilibrium dissociation constants calculated from the concentration dependence of $\Delta\pi$ indicated that the affinity of prothrombin for LE monolayers was higher at higher PS contents and lower packing densities. The affinity of prothrombin for liquid-condensed (LC) PC/PS monolayers was found to be much weaker relative to LE monolayers of similar phospholipid composition. This approach, employing spread monolayers to study prothrombin-phospholipid binding, coupled with a simple and accurate method to determine the free protein concentration in protein-monolayer suspensions, offers significant advantages for the investigation of protein-membrane interaction. The equilibrium characteristics that describe the interaction of prothrombin with the different phospholipid monolayers under various conditions also provide support for previous results which indicated that hydrophobic interactions are involved in the adsorption of vitamin K-dependent coagulation and anticoagulation proteins to model membrane systems.

INTRODUCTION

The adsorption of γ-carboxyglutamic acid (Gla)-containing plasma proteins to cell membranes, such as those of platelets (Swords and Mann, 1993), peripheral blood mononuclear cells (Tracy et al., 1983), and endothelial cells (Rodgers and Shuman, 1983), is critical for the proper functioning of the blood coagulation cascade and its regulatory pathways. Strong evidence exists that the adsorption of these proteins to membranes is driven by a Ca^{2+}-mediated bridging interaction between negatively charged phospholipid (e.g., phosphatidylserine) and the Gla residues of proteins (for a review, see Mann et al., 1990). More recent evidence was interpreted to demonstrate that the adsorption of coagulation factors VII, IXa, and X (Atkins and Ganz, 1992), prothrombin (Lecompte and Dode, 1992), and anticoagulant protein C (Zhang and Castellino, 1992; Christiansen et al., 1995; Jalbert et al., 1996) to phospholipid monolayers involved a significant hydrophobic component. Such an interaction has been described as the penetration of a small hydrophobic region of the protein into the hydrocarbon region of the membrane (Zhang and Castellino, 1994; Christiansen et al., 1995; Jalbert et al., 1996). In the case of human protein C, major contributors to the hydrophobic component of this interaction were found to reside at amino acids Phe^4, Leu^5, and Leu^8 (Zhang and Castellino, 1994; Christiansen et al., 1995), residues that are either invariant or highly conserved in Gla-containing proteins of this class. Furthermore, in the presence of Ca^{2+}, these amino acid residues are clustered in a solvent-exposed area of the protein and are well separated from the majority of other hydrophobic amino acids of the Gla-domain, which accumulate in a region of the protein that is largely unexposed to solvent (Soriano-Garcia et al., 1992; Christiansen et al., 1995; Banner et al., 1996).

Most studies of blood coagulation protein-membrane interactions have been accomplished with mixed lipid vesicle bilayer systems. Such strategies are complicated by the fact that changes in bulk lipid compositions in these systems also have profound effects on biophysical properties of the bilayer, such as its radius of curvature and its inner-outer membrane compositional symmetry. Furthermore, such work is limited by the inabilities of certain phospholipids to assume a bilayer structure, and/or to disrupt such a bilayer system when it does exist. These biophysical characteristics of the bilayer can influence protein binding, independent of the effects of the bulk chemical composition of the lipid system used to form the bilayer. Because of these potential problems with bilayer-based investigations, we have examined phospholipid monolayers spread at the air-water interface below their collapse pressures as model systems to study the properties of protein-lipid interactions. This approach has been successfully employed in the past in systems of importance to this work, wherein the interactions between prothrombin fragment 1 and spread monolayers composed of egg phosphatidylcholine (ePC) and bovine brain phosphatidylserine (bPS) have been examined (Mayer et al., 1983a). This experimental design offers significant

Received for publication 15 October 1996 and in final form 11 March 1997.

Address reprint requests to Dr. Francis J. Castellino, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556. Tel.: 219-631-6456; Fax: 219-631-8017; E-mail: castellino.1@nd.edu.

0006-3495/97/06/2605/11 $2.00

advantages over a bilayer system for such work, specifically because 1) the packing densities can be varied at constant area; 2) a full range of PS compositional effects can be examined without concern for their abilities to form bilayers and/or to influence the inner-outer membrane composition of a bilayer; and 3) because measurements of the influence of adsorbed proteins on the surface tension of the monolayer at the lipid-air interface can yield unique information regarding the binding energies. Regarding this latter point, measurements of the surface tensions of bilayers and the effects of proteins thereon are not directly possible with bilayer systems, because the surface tension of bilayer membranes is zero (Israelachvili et al., 1977; Jahnig, 1996). In the current study we have employed mixed phospholipid spread monolayer systems to study the interaction of bovine prothrombin with both disaturated and unsaturated synthetic phospholipids, and to more fully explore the applicability of this system to investigations of the interaction of intact coagulation proteins with lipids. The results of this investigation are described herein.

MATERIALS AND METHODS

Materials

The phospholipids, ePC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS), were obtained from Avanti Polar Lipids (Alabaster, AL). Lipid concentrations were determined by weighing known volumes of lipid solutions after evaporation of the solvent under vacuum. Buffer components and spreading solvents were reagent grade or better. The principal buffer used was 10 mM borate/100 mM NaCl/10 mM $CaCl_2$ (pH 8.0). Buffers not containing free Ca^{2+} contained 0.5 mM EDTA. Filtered, deionized H_2O was obtained from a Milli-Q UV-plus delivery system.

Adsorbates

Bovine prothrombin (molecular weight 70,000) was obtained from Enzyme Research (South Bend, IN) and subsequently dialyzed to remove benzamidine. Its purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein concentration was determined by absorption spectroscopy (ϵ_{280} = 1.44 ml mg^{-1} cm^{-1}). 1-Pyrenebutyltrimethylammonium bromide (PBN) was obtained from Molecular Probes (Eugene, OR).

Apparatus

The surface pressure (π) was measured with the use of a Cahn 2000 electrobalance. A 1.2 cm × 1.0 cm^2 section of filter paper (Whatman no. 50) was employed as the Wilhemly plate (Gaines, 1977). Surface pressure measurements were made with the Wilhemly plate in a fixed-height position. Pressure (π)-area (A) curves of phospholipids were collected at a compression speed of 0.10 $Å^2$ mol^{-1} min^{-1} after spreading of the lipid on a 700-cm^2 buffer surface contained in a rectangular Teflon trough. Before the lipid was spread, surface active contaminants that may be present on the buffer surface were collected in a small area by using a hand-held Teflon barrier and were removed by aspiration of the surface.

A 30-ml circular trough with a surface area of 20 cm^2 was constructed locally from commercial quartz and used to measure protein adsorption to the monolayers. A capillary tube was fused to the quartz trough to allow for the injection of solutions into the subphase. The quartz trough was cleansed with a mild detergent solution and rinsed with copious amounts of ethanol and water between experiments.

Monolayers

The π-A curves of various PS lipid monolayers have been thoroughly examined (Demel et al., 1987). These studies indicated that, at room temperature, POPS and bPS produce liquid expanded (LE) films and similar π-A curves. In this way, we compared the π-A curves of ePC and POPC. These phospholipids also produced LE films and similar π-A curves. Therefore, POPC and POPS were selected as the principal lipids in our examination of adsorption of prothrombin to monolayers, because they more closely mimicked the behavior of natural membrane extracts. The use of synthetic lipids allowed control of the hydrocarbon properties, such as chain length and degree of unsaturation, so that strict comparisons of different phospholipid systems were possible. With the use of POPC and POPS, phase separation, due to differences in degree of chain unsaturation, cannot occur. In the presence of Ca^{2+}, PS-PS headgroup interactions are more favorable (or less repulsive), and this results in a lowering of the surface pressure. The stoichiometry of the PS/Ca^{2+} complex in 1,2-dioleoyl-sn-glycero-3-phospho-L-serine, (DOPS), POPS, and bPS was determined to be 2/1 (Feigenson, 1986).

Measurements of prothrombin adsorption at monolayer surfaces

Monolayers for protein analysis were prepared by carefully spreading one drop (4–5 μl) of lipid solution in chloroform on the buffer surface (for numerous examples of the use of chloroform in this regard, see Mingotaud et al., 1996). Spreading and stabilization of the surface pressure were achieved equally well using either chloroform or ethanol/hexane (10:90, v:v) as the spreading solvent. After the film pressure stabilized at a constant value, the protein was injected into the subphase. Prothrombin was equilibrated in buffer containing 10 mM Ca^{2+} for at least 2 h before experiments that included Ca^{2+} in the subphase.

The adsorption of prothrombin to monolayers was assessed from measurements of $\Delta\pi$ and the change in its bulk subphase concentration (Δc_b). Fluorescence spectroscopy was employed to measure Δc_b (λ_{ex} = 280 nm, λ_{em} = 335 nm). Simultaneous measurements were made of $\Delta\pi$ and the drop in subphase fluorescence intensity. This was achieved by constructing an apparatus specially designed and locally constructed for an SLM-Aminco 8000 ratio spectrofluorometer. The apparatus essentially consisted of an inert atmosphere chamber in which the quartz trough was appropriately positioned for the fluorescence measurements. Nonevasive stirring (<120 rpm) of the subphase, or stirring that does not disturb the monolayer, was used during the course of all adsorption measurements.

The change in subphase fluorescence intensity due to adsorption varied between 5% and 15%. To determine Δc_b, accurate values of the initial fluorescence intensity were required. With stirring of the subphase, the fluorescence intensity stabilized within 2–3 min of injection. After this mixing period, the intensity decreased, depending on the nature of the film and c_b. At $c_b < 0.05$ μM, the drop in intensity was sufficiently slow that the initial fluorescence intensity could be determined by extrapolation to time 0. Because the fluorescence intensity was observed to be proportional to c_b (at the low protein concentrations used in this study), and the background fluorescence was negligible, the protein concentration at the monolayer surface (c_s) was determined from the percentage change in fluorescence intensity with the aid of standard curves of fluorescence intensity versus prothrombin concentration.

RESULTS

Interaction of prothrombin with POPC/POPS monolayers

When the bulk concentration (c_b) of prothrombin in the subphase of POPC/POPS monolayers was sufficiently low, the adsorption of prothrombin to the monolayer produced a measurable decrease in c_b. This was determined from time-resolved measurements of the subphase fluorescence intensity after the injection of prothrombin into the subphase. The adsorption of prothrombin to monolayers was always accompanied by an increase in π. We restricted our analysis to initial surface pressures (π_i) < 40 mN/m, which was found to be less than the collapse pressure of any of the pure or mixed films.

Fig. 1 *A* (*curve b*) shows the time dependence of the fluorescence intensity (dI_f/dt) in the subphase of a 1:2 (m:m) POPC/POPS LE monolayer, when c_b = 0.040 μM immediately after injection. Fig. 1 *B* exhibits the corresponding profile of $d\pi/dt$. The baseline fluorescence intensity was monitored under conditions in which adsorption at the air-buffer interface was inhibited. This was achieved by spreading a DPPC monolayer at the interface to yield a surface pressure above the LE-LC phase coexistence region (e.g., at π_i = 35 mN/m). The high rigidity of the DPPC film most probably inhibits those hydrophobic protein-monolayer interactions that would require a penetration of the monolayer. In addition, electrostatic interactions between prothrombin and the zwitterionic PC headgroup are relatively weak or nonexistent. As indicated in Fig. 1 *A* (*curve a*), the change in fluorescence intensity below the DPPC film was much less relative to the 1:2 (m:m) POPC/POPS LE monolayer. Furthermore, there was also no change in π of the DPPC monolayer. The small decrease in fluorescence intensity that was observed is probably due to photodegradation or possibly to adsorption of prothrombin to the quartz surface.

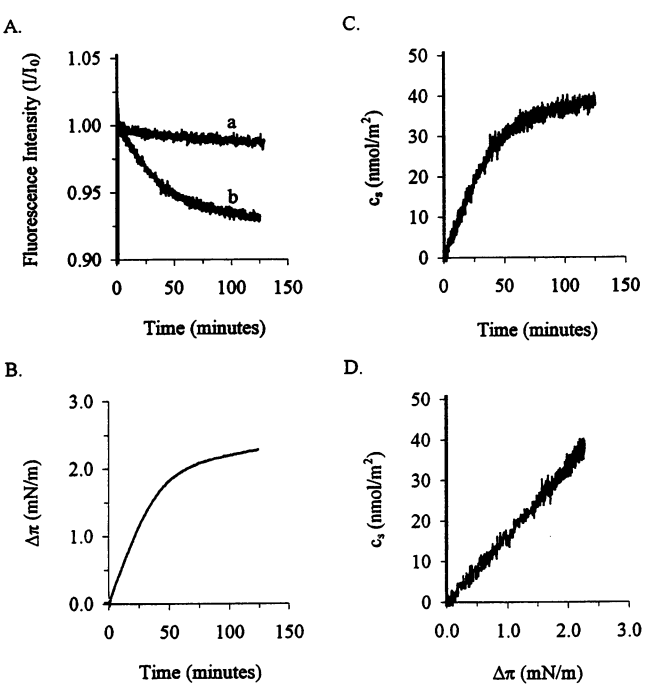

FIGURE 1 Response of the surface pressure and subphase fluorescence intensity to the adsorption of bovine prothrombin to spread monolayers. (*A*) The decrease in subphase relative fluorescence intensity (I/I_o) in a DPPC monolayer (*curve a*) and 1:2 (m:m) POPC/POPS monolayer (*curve b*). (*B*) The increase in surface pressure ($\Delta\pi$) due to adsorption of prothrombin to a 1:2 (m:m) POPC/POPS monolayer at π_i = 34 mN/m. (*C*) The increase in concentration of prothrombin (c_s) adsorbed to the monolayer. (*D*) Plot of c_s versus $\Delta\pi$ used to determine $dc_s/d\pi$. The final concentration of prothrombin added to the aqueous phase was 0.040 μM. The buffer was 10 mM borate/100 mM NaCl/10 mM $CaCl_2$ (pH 8.0). The temperature was 25°C.

A linear regression of dI_f/dt in the DPPC subphase was used as the baseline fluorescence intensity. Fig. 1 *C* illustrates the increase in concentration (c_s) of prothrombin adsorbed to the 1:2 (m:m) POPC/POPS LE monolayer, as determined from the percentage change in fluorescence intensity. Under conditions in which the decrease in fluorescence intensity was too small to be measured accurately (e.g., at high c_b), c_s was determined from measurements of $\Delta\pi$. This required values of $dc_s/d\pi$ (nmol m^{-1} mN^{-1}), or the c_s that produces a one-unit increase in π. Values of $dc_s/d\pi$ were determined at low c_b, where there was a measurable increase in π and a decrease in the fluorescence intensity. This condition was generally satisfied at c_b < 0.050 μM. However, at c_b < 0.020 μM, the approach to equilibrium was slow and measurement times were too long to be practical. Because $dc_s/d\pi = (dc_s/dt)(dt/d\pi)$, $dc_s/d\pi$ is equivalent to the slope of $c_{s,t}$ versus π_t. This is illustrated in Fig. 1 *D*. The linear response in Fig. 1 *D* and the similar time scales of the processes in Fig. 1, *A* and *C*, indicate that $\Delta\pi$ is simultaneous with adsorption, or that $\Delta\pi$ is not a significantly delayed process. The fact that $dc_s/d\pi$ is constant throughout the adsorption implies that prothrombin behaves essentially as a solute in its influence on the monolayer, i.e., the rise in pressure from a given number of adsorbed prothrombin molecules is similar, regardless of whether prothrombin has previously adsorbed to the monolayer.

Using the approach described above, the dependence of $dc_s/d\pi$ on π_i and PS content of the POPC/POPS LE monolayer was evaluated. The values are given in Table 1. In the region π_i > 20 mN/m, $dc_s/d\pi$ was nearly constant at a particular PS content. However, a higher PS ratio led to lower values of $dc_s/d\pi$, or to a greater sensitivity of $\Delta\pi$ to c_s.

TABLE 1 Influence of π_i and PS content of spread monolayers on $c_{s,e}$ and $dc_s/d\pi$ at 25°C

2:1 (m:m) POPC/POPS			1:2 (m:m) POPC/POPS		
π_i (mN/m)	$c_{s,e}$ (nmol/m^2)	$dc_s/d\pi$ (mN-m/nmol)	π_i (mN/m)	$c_{s,e}$ (nmol/m^2)	$dc_s/d\pi$ (mN-m/nmol)
24	32	20	24	41	14
29	29	23	30	39	17
37	10	24	34	38	17

The data were obtained by injecting prothrombin into the subphase, so that the initial c_b = 0.040 μM. The buffer was 10 mM borate/100 mM NaCl/10 mM $CaCl_2$, pH 8.0.

π-A curves of the mixed PL monolayers were also examined to determine values of $dc/d\pi$ for the lipid monolayers in the region 25–40 mN/m. Within this pressure range, the π-A curves were linear. Plots of $1/A$ versus π were also linear and yielded slopes of 22.7 and 22.5 (nmol/m^2)/(mN/m) for 2:1 and 1:2 (m:m) POPC/POPS monolayers, respectively. These values are very similar to those in Table 1. This indicates that, at constant area, one prothrombin molecule adsorbed to the film produces a $\Delta\pi$ roughly equivalent to adding one lipid molecule to the same film.

At $\pi_i < 20$ mN/m, plots of $c_{s,t}$ versus π_t were not linear. This resulted from a nonspecific interaction between adsorbed prothrombin and the monolayer. The nonspecific interaction is best described by the increase in π that results from the adsorption of prothrombin to pure POPC monolayers. This is illustrated in Fig. 2. At higher c_b and lower π_i, $d\pi/dt$ of the POPC monolayer was larger. In all likelihood, the change in $d\pi/dt$ due to π_i reflected a difference in dc_s/dt. This assumes that $dc_s/d\pi$ is constant with π_i and that the slope of the π-A curve of the POPC film does not vary substantially over the pressure range studied. We have observed the latter to be true. With regard to the former, the response of the fluorescence intensity in the subphase of POPC films was indistinguishable from that of DPPC films. In addition, the adsorption of prothrombin to the POPC monolayer was slow and equilibrium measurements were not feasible within a few hours. This problem was more severe at film pressures closer to physiological conditions, i.e., at or above 30 mN/m, a pressure at which the monolayer produces results similar to those of the erythrocyte membrane (Demel et al., 1975). Therefore, the dependence of c_s and $dc_s/d\pi$ on π_i of POPC monolayers could not be evaluated.

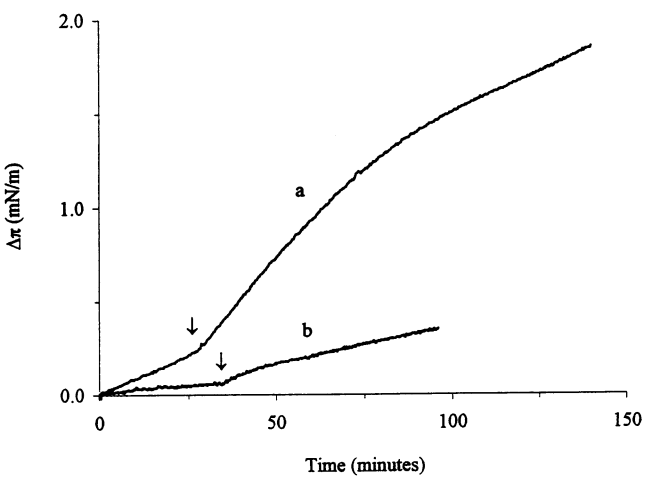

FIGURE 2 Dependence on initial surface pressure (π_i) of the adsorption of bovine prothrombin to POPC monolayers at 25°C. The profiles of change in surface pressure ($\Delta\pi$) versus time resulting from two injections of prothrombin below POPC monolayers (↓ , times of second injections) at π_i = 16 mN/m (*curve a*) and 23 mN/m (*curve b*). The final concentrations of prothrombin added to the aqueous phase were 0.04 μM (after injection 1) and 0.12 μM (after injection 2). The buffer was 10 mM borate/100 mM NaCl/10 mM $CaCl_2$ (pH 8.0).

The increase in π of POPC monolayers could involve the insertion of a single segment of prothrombin, but it could also involve many segments, or even the entire molecule. At this stage we are uncertain of the exact features of the penetration of prothrombin into the POPC monolayer. Significant interaction of prothrombin with POPC has been observed in a previous study employing self-assembled planar POPC bilayers on glass substrates (Tendian et al., 1991). However, the affinity of prothrombin for the POPC bilayer was too weak to be of significance in affecting the Ca^{2+}-specific binding to mixed bilayers containing POPC and negatively charged bovine brain PS or 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)](DOPG).

The protein-monolayer interaction that produced the adsorption of prothrombin to POPC monolayers also occurred between prothrombin and POPC/POPS monolayers. Adsorption of prothrombin to POPC monolayers is a dynamic process. As π_i increases, the number of molecules arriving at the interface with the appropriate activation energy for a penetration decreases exponentially (MacRitchie, 1990). However, when POPS is present at the interface, there is a much higher protein concentration at the interface because of the PS-specific interaction. This should increase the probability of the nonspecific interaction. It is certainly possible that the adsorbed prothrombin molecules interact with POPC/POPS monolayers in the same way that prothrombin interacts with POPC monolayers. As indicated with POPC monolayers, penetration of the monolayer is more facile and $\Delta\pi$ is larger as π_i decreases. A similar effect of π_i on $\Delta\pi$ was also observed when prothrombin was adsorbed to POPC/POPS monolayers (Fig. 3). At lower π_i, $d\pi/dt$ (Fig. 3 *A*) is larger at longer times, but dI_f/dt (Fig. 3 *B*) in the same region does not vary with π_i. A similar result was observed when 1:2 POPC/POPS LE monolayers were compared.

Two processes appear to contribute to the prothrombin-mediated rise in π of POPC/POPS monolayers. One produces the rapid increase in π and the decrease in fluorescence intensity and is due to the PS-specific interactions. The other produces a much slower increase in π and is the result of the nonspecific interaction observed in POPC monolayers. The influence of the nonspecific interaction was considerable at $\pi_i < 20$ mN/m, and this produced the nonlinearity of $c_{s,t}$ versus π_t. At $\pi_i > 30$ mN/m, the nonspecific interaction does not significantly interfere with the analysis of $dc_s/d\pi$.

Visual inspection of Fig. 3 *B* reveals a rapid and a slow component of the protein-monolayer adsorption profile. None of the POPC/POPS monolayers exhibited a leveling of c_s within 4 h after injection of prothrombin into the subphase. We are uncertain whether the slow component is due to actual adsorption or to some unknown background process associated with the interaction between prothrombin and the POPC/POPS monolayer. The increase in π in the latter stages of the profiles in Fig. 3 *A* is due, in part, to the nonspecific interaction, but could also result from slowly adsorbing components.

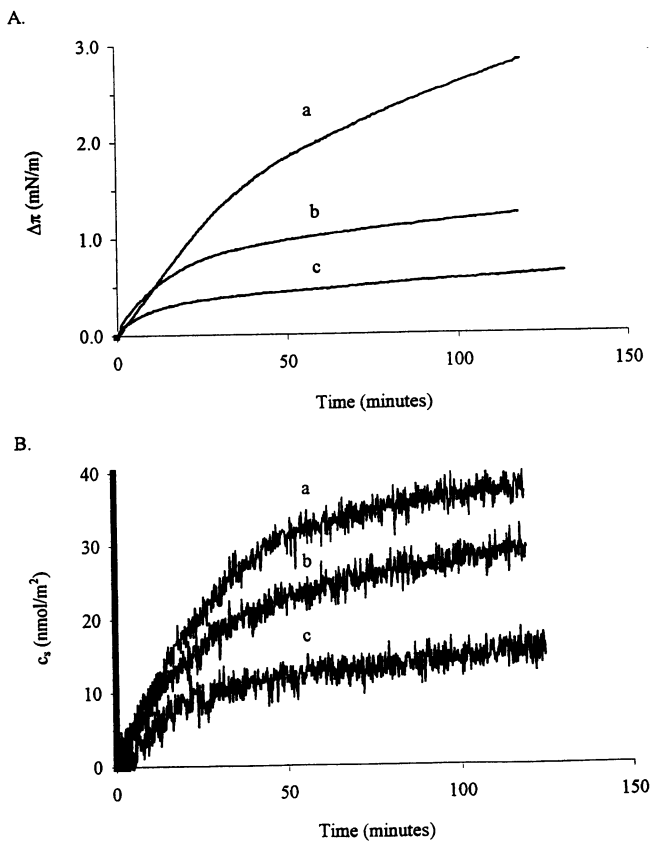

FIGURE 3 Adsorption of prothrombin to LE monolayers at 25°C. (*A*) The change in surface pressure ($\Delta\pi$) of a 2:1 (m:m) POPC/POPS monolayer due to adsorption of prothrombin. (*B*) The corresponding increase in prothrombin concentration adsorbed (c_s) to these same monolayers. The initial concentration of prothrombin was 0.040 μM. π_i = 16 mN/m (*a*), 29 mN/m (*b*), and 36 mN/m (*b*). The buffer was 10 mM borate/100 mM NaCl/10 mM $CaCl_2$ (pH 8.0).

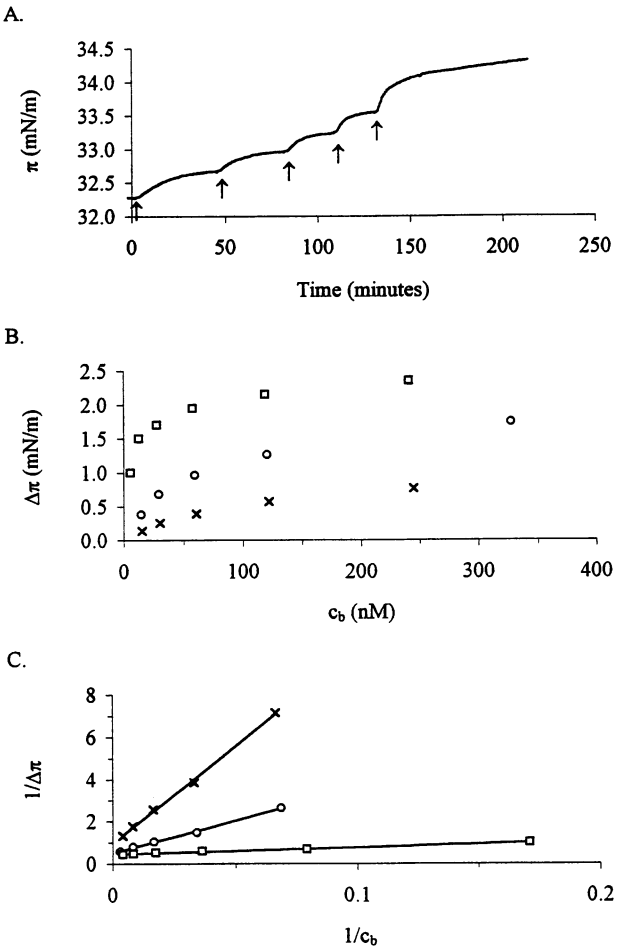

FIGURE 4 Dependence on initial surface pressure (π_i) and PS content of the adsorption of bovine prothrombin to POPC/POPS LE monolayers at 25°C. (*A*) Representative plot of the change in monolayer surface pressure ($\Delta\pi$) from successive additions (↓ , injection points) of prothrombin in the aqueous subphase of a 2:1 (m:m) POPC/POPS monolayer. (*B*) Adsorption isotherms of the binding (c_b = prothrombin concentration). (*C*) Double-reciprocal plots of the adsorption isotherms. □, 1:2 (m:m) POPC/POPS film at π_i = 37 mN/m; ○, 2:1 (m/m) POPC/POPS film at 32 mN/m; ×, 2:1 (m/m) POPC/POPS film at 38 mN/m. The buffer was 10 mM borate/100 mM NaCl/10 mM $CaCl_2$ (pH 8.0).

The dependence of K_d on π_i and PS content of POPC/POPS monolayers

Adsorption isotherms and K_d values were assessed from measurements of $\Delta\pi$ at various c_b. A representative plot is illustrated in Fig. 4 *A*. It is evident in Fig. 4 *A* that the equilibration time is smaller at higher c_b. Plots of $\Delta\pi$ versus c_b are illustrated in Fig. 4 *B*. The values of $\Delta\pi$ used in Fig. 4 *B* were those from the rapid increase in π; the slow $\Delta\pi$ was ignored. Those for c_b in Fig. 4 *B* were corrected for the decrease in c_b due to adsorption by using values of $\Delta\pi$ and $dc_s/d\pi$, but this did not markedly change the results. The corresponding double reciprocal plots of these data are illustrated in Fig. 4 *C*, from which the dependence of K_d on PS content and π_i was calculated. To accurately estimate c_s at or near saturating subphase concentrations (or $c_{s,sat}$), prothrombin was injected into the subphase, so that the final concentration of prothrombin added to the aqueous subphase was at least 10-fold in excess of the estimated K_d. $\Delta\pi$ was determined from the data and converted to $c_{s,sat}$ by employing the average value of $dc_s/d\pi$ in Table 1. In determining values of $c_{s,sat}$ from $\Delta\pi$, the assumption was made that each adsorbed prothrombin molecule produces a $\Delta\pi$ identical to those of the others, regardless of c_s.

Values of $c_{s,sat}$ were not highly dependent on π_i and PS content. At π_i = 28 mN/m and 37 mN/m of 2:1 (m:m) POPC/POPS LE monolayers, $c_{s,sat}$ values for prothrombin of 58 nmol/m^2 and 53 nmol/m^2, respectively, were obtained. At π_i = 28 mN/m and 37 mN/m of 1:2 (m:m) POPC/POPS LE monolayers, the $c_{s,sat}$ values for prothrombin were 56 nmol/m^2 and 54 nmol/m^2, respectively. The similarities of the values imply that there is a steric limitation to the adsorption of prothrombin to monolayers. The values of $c_{s,sat}$ in similar systems vary somewhat in the literature, but this could be due to calibration errors. However, those determined here are not very different from previously published data (see table 1 in Kop et al., 1984). According to the average value (55 nmol/m^2) of $c_{s,sat}$ determined here,

the surface area occupied by prothrombin on the monolayer is 3000 Å^2/molecule.

From titrations of prothrombin to 2:1 (m:m) POPC/POPS LE monolayers, K_d values of 0.02 μM, 0.06 μM, and 0.1 μM were obtained at π_i values of 25 mN/m, 32 mN/m, and 38 mN/m, respectively. At 37 mN/m, a 1:2 (m:m) POPC/POPS LE monolayer yielded a K_d of 0.007 μM. These values indicate that the affinity of prothrombin for the monolayer containing the larger relative amount of POPS is approximately an order of magnitude greater than for the monolayer containing the lower relative amount of POPS. Furthermore, the K_d values suggest that the affinity of prothrombin for the 2:1 (m:m) POPC/POPS monolayer decreases with increasing π_i. In the case of the 1:2 (m:m) POPC/POPS monolayer, we were unable to establish any differences in K_d values due to π_i. This was mainly because very low c_b produced a significant $\Delta\pi$ of 1:2 (m:m) POPC/POPS monolayers. For example, at c_b = 10 nM prothrombin, a $\Delta\pi$ = 1 mN/m was produced over the course of 2 h. Measurements of $\Delta\pi$ at c_b < 10 nM were not feasible because the approach to equilibrium was too slow. To more accurately estimate the slope and intercept of double-reciprocal plots, data points at lower subphase concentrations than K_d are required. Therefore, the value of K_d for 1:2 (m:m) POPC/POPS monolayers is subject to more error than at the lower PS content. Nevertheless, the data certainly indicate that the affinity of prothrombin for LE (POPC/POPS) increases with increasing PS content and lower π_i of the monolayer.

Because of the uncertainty in measuring K_d at the higher PS content (i.e., 1:2 POPC/POPS monolayer), we were not able to rely on values of $\Delta\pi_{s,sat}$ determined from Fig. 3 *C*. It was for this reason that $\Delta\pi_{s,sat}$ was estimated by injecting an excess of prothrombin into the subphase. However, the measured values of $\Delta\pi_{s,sat}$ used to calculate values of $c_{s,sat}$, listed above, are in close agreement with estimated values of $\Delta\pi_{s,sat}$ determined from the double-reciprocal plots corresponding to adsorption of prothrombin to 2:1 POPC/POPS monolayers (see Fig. 3 *C*). Values of $c_{s,sat}$, calculated from the $\Delta\pi_{s,sat}$ of Fig. 3 *C* and $dc_s/d\pi$ = 24, were 55 and 48 nmol/m^2 at π_i = 37 and 32 mN/m, respectively.

Interaction of prothrombin with POPC/POPS monolayers in the absence of Ca^{2+}

The interaction of prothrombin with POPC/POPS monolayers in the absence of Ca^{2+} (or in the presence of EDTA) was also examined. In the absence of Ca^{2+}, the monolayer is less condensed at a given pressure and prothrombin is in a conformationally different state. These conditions produced essentially the same results as those in Fig. 2, when POPC monolayers were used. This indicates that the negatively charged interface in itself does not allow significant adsorption of the Ca^{2+}-free protein.

Interaction of prothrombin with DPPC/DPPS monolayers

The adsorption of prothrombin to DPPC/DPPS LC monolayers below the phase transition temperature (T_m) was investigated to assess the influence of film rigidity on prothrombin binding. In general, when a penetration of the monolayer is involved in the adsorption, LC monolayers should promote adsorption less effectively than LE monolayers.

Our observations indicate that the affinity of prothrombin for LC monolayers was substantially less relative to LE monolayers. Injection of 0.040 μM prothrombin into the subphase of either 2:1 (m:m) DPPC/DPPS or 1:2 DPPC/DPPS LC monolayers at a π_i value of 30 mN/m did not produce a significant increase in π. Although there could be adsorption without a rise in π, this was not observed. The fluorescence intensity profiles were indistinguishable from those of DPPC monolayers.

At c_b > 0.040 μM, there was a measurable increase in π of 2:1 DPPC/DPPS LC monolayers, and the rate of increase in π was smaller at lower c_b. This is illustrated in Fig. 5. Equilibrium measurements were not feasible, because of the slow approach to equilibrium.

Injection of EDTA below a 2:1 DPPC/DPPS LC monolayer, at a level sufficient to complex all of the free Ca^{2+}, produced a 10 mN/m increase in π within 30 min at π_i = 30 mN/m. This indicates that the LC surface is somewhat negatively charged and capable of adsorbing Ca^{2+}. A similar effect was observed when we used 2:1 POPC/POPS LE monolayers, although the rise in π was only 2 mN/m. The difference is due to the steeper slope of the π-*A* curve of the LC monolayer and the larger number of PS molecules in the LC monolayer.

Interaction of PBN with LE and LC monolayers

To illustrate the general dependence of adsorption on film rigidity, the interaction of a small molecule (PBN) with LC

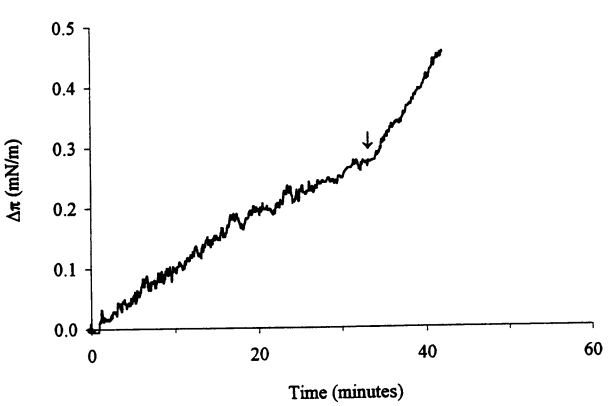

FIGURE 5 Adsorption of bovine prothrombin to DPPC/DPPS monolayers at 25°C. The change in surface pressure ($\Delta\pi$) resulting from two injections (↓, time of second injection) of prothrombin below a 2:1 (m:m) DPPC/DPPS monolayer at π_i = 19 mN/m. The final concentrations of prothrombin added to the aqueous subphase were 0.15 μM (after injection 1) and 0.60 μM (after injection 2). The buffer was 10 mM borate/100 mM NaCl/10 mM $CaCl_2$ (pH 8.0).

and LE monolayers was compared. PBN, by virtue of its butyltrimethylammonium ion, is not only able to interact electrostatically with negatively charged phospholipid headgroups, but can also interact with the monolayer through hydrophobic-type interactions. The latter requires an insertion of the pyrenyl group into the hydrocarbon region of the monolayer. PBN is also more water soluble than other pyrene derivatives because of its high degree of charge density, thus enhancing its suitability for such studies.

To assess the adsorption of PBN to monolayers, it was first necessary to characterize its affinity for the trough surface. Therefore, the subphase fluorescence intensity and $\Delta\pi$ were examined in the presence of a spread DPPC monolayer. At a subphase concentration of 0.042 μM PBN, no rise in π of the DPPC monolayer was observed over the course of 2 h. The upper curve in Fig. 6 *A* represents the fluorescence intensity in the subphase of the DPPC monolayer. The small decrease in fluorescence is probably due to adsorption of PBN to the quartz surface. This is expected, because the quartz surface silanols are negatively charged at pH 8.0.

The addition of PBN to the aqueous subphase of a 1:2 (m:m) POPC/POPS LE monolayer led to the results in Fig. 6, *A* (*curve b*) and *B*. Free Ca^{2+} was excluded from the subphase, so that PBN was able to interact in an unimpeded fashion with the negatively charged surface. There was a much larger drop in the fluorescence intensity relative to the DPPC monolayer and a measurable increase in π. To evaluate the influence of POPS on adsorption of PBN to LE monolayers, adsorption of PBN to a pure POPC monolayer was examined at π_i = 18 mN/m and c_b = 0.042 μM. No significant adsorption was observed. Measurements of the subphase fluorescence were indistinguishable from the results obtained for DPPC monolayers, and there was no significant rise in π.

The affinity of PBN for a 1:2 (m:m) DPPC/DPPS LC monolayer at π_i = 30 mN/m was also weak; the results were similar to those obtained from DPPC monolayers. Because the negative charge density of the 1:2 DPPC/DPPS LC monolayer is high, the hydrophobic interaction must be inhibited by the high packing density.

These results indicate that adsorption of PBN to monolayers is enhanced by a hydrophobic interaction with the monolayer and by a favorable electrostatic interaction. The electrostatic interaction should be more favorable if water is used as the subphase. A recent study indicated that the affinity of PBN for negatively charged quartz surfaces is very strong, but considerably weakened at 0.10 M ionic strength (Ellison and Thomas, 1996). At the quartz surface, adsorption does not involve hydrophobic interactions. This indicates the potential for PBN adsorption to negatively charged LC monolayers without the involvement of hydrophobic interactions.

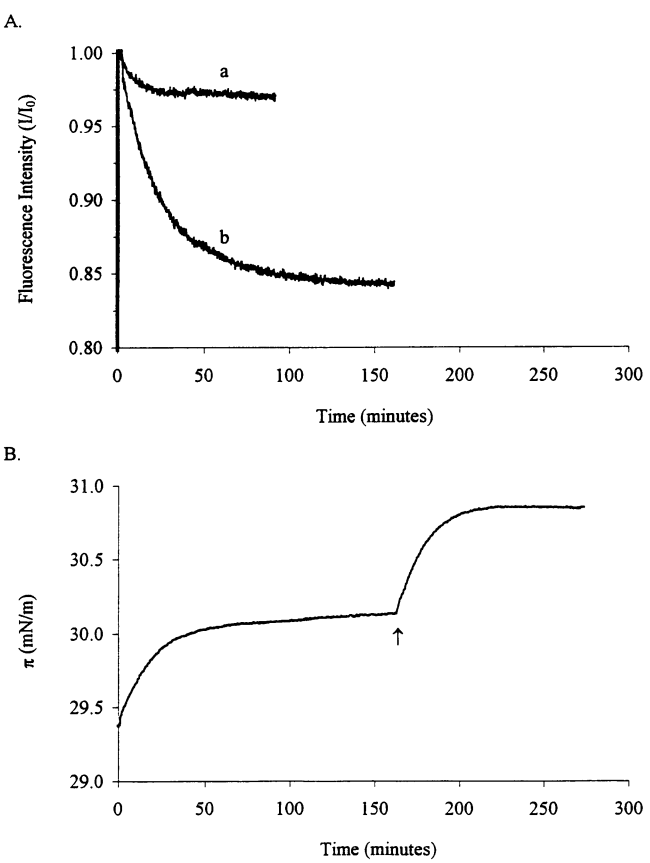

FIGURE 6 Adsorption of PBN to spread monolayers. (*A*) The decrease in subphase fluorescence intensity (I/I_o) due to adsorption of PBN to a DPPC LC film at π_i = 35 mN/m (*curve a*), and a 1:2 (m:m) POPC/POPS film at π_i = 29 mN/m (*curve b*). The concentration of PBN added to the aqueous subphase was 0.042 μM. (*B*) The corresponding increase in surface pressure (π) of the 1:2 (m:m) POPC/POPS LE monolayer due to the adsorption of PBN. Graph B includes a second addition (at the time indicated by ↓), a PBN concentration of 0.084 μM. The buffer was 10 mM borate/100 mM NaCl/0.5 mM EDTA (pH 8.0).

DISCUSSION

We have developed an approach for measuring the concentration of prothrombin adsorbed to spread phospholipid monolayers that is generally applicable to most proteins, viz., by directly and continually measuring the decrease in the subphase concentration of the protein in the presence of various types of spread monolayers. One advantage of our strategy is that radiolabeled protein is not needed. In addition, it does not require transfer of the monolayer and adsorbed protein to solid substrates (such as hydrophobic filter paper), where the transfer efficiency and its dependence on π are unknown. Furthermore, in our case, the increase in adsorbed protein concentration can be monitored during the approach to equilibrium. The values of $dc/d\pi$ obtained from this experimental design allow the measurement of adsorbed protein concentrations from measurements of π under conditions in which the change in subphase fluorescence intensity (or protein concentration) is not measurable. We have found that $\Delta\pi$ reflects the increase in adsorbed concentration, and we did not observe signifi-

cant adsorption of prothrombin to monolayers without a change in π.

Spread phospholipid monolayers have previously been used to examine the prothrombin-membrane interaction. It has been determined from studies employing a radioactively labeled prothrombin that $K_d = 0.070$ μM for the interaction of prothrombin with 4:1 (m:m) ePC/bPS monolayers at $\pi_i = 24$ mN/m (Mayer et al., 1983a). However, this study focused on prothrombin fragment 1 adsorption, and only one value of K_d was determined for prothrombin. Nevertheless, it is close to the values obtained in this study using 2:1 POPC/POPS monolayers (see Table 1). As previously determined (Mayer et al., 1983a), the K_d of prothrombin fragment 1 was higher relative to prothrombin and exhibited little dependence on the PS content of the monolayer. For example, 4:1 and 1:4 ePC/bPS LE monolayers at $\pi_i \approx 21$ mN/m exhibited K_d values of 0.15 μM and 0.11 μM, respectively. We have found that K_d of the prothrombin-monolayer interaction is strongly dependent on the PS content of the monolayer and varies by an order of magnitude when 2:1 and 1:2 POPC/POPS LE monolayers are compared at similar π_i. Evidently, there are differences between prothrombin and fragment 1 in their association with spread phospholipid monolayers. This indicates that, in addition to the prothrombin fragment 1 domain, other protein domains could be involved in the adsorption dynamics, and/or that the remainder of the prothrombin molecule is needed for full expression of the membrane-binding site in the fragment 1 region of this protein. There was no indication of a nonspecific interaction between fragment 1 and spread monolayers. Perhaps the nonspecific interaction is somehow involved in the adsorption dynamics. For example, it may increase the encounter time at the surface, for more effective searching by prothrombin for PS.

The K_d values describing the interaction of prothrombin with PC/PS vesicles vary widely in the literature. A representative K_d of 1 μM for the interaction of prothrombin with liposomes, at PS contents that are not limiting, has been estimated (for a review, see Mann et al., 1990). Nelsestuen and Lim (1977) determined from equilibrium light scattering studies that $K_d = 0.3$ μM for the interaction of prothrombin with PC/PS small unilamellar vesicles (SUVs) containing 40% (m:m) PS. Later studies (Wei et al., 1982) confirmed this value. However, a recent stopped-flow kinetic investigation that employed a fluorescence energy transfer approach to make direct measurements of the association and dissociation rate constants for the interaction of prothrombin with PC/PS vesicles resulted in different values for the binding of prothrombin to these vesicles (Lu and Nelsestuen, 1996). From this latter work, K_d values of 0.008 μM and 0.1 μM for the interaction of prothrombin with SUVs containing 50% and 25% PS, respectively, have been obtained. It is unclear to us why these results are different from the earlier ones. Further work may be necessary to account for these differences and to clarify the limitations of the vesicle/light-scattering methodology.

Relative to the vesicle approach, the use of monolayers as the phospholipid source provides a less ambiguous interpretation of the relative affinity of prothrombin for organized lipid assemblies that vary in PS content, in packing density, and in degree of fatty acid chain unsaturation. It also allows manipulation of more variables to examine more fully the protein-monolayer interaction, e.g., the lipid packing density in the monolayer can be readily varied. The use of vesicles requires a change in vesicle size to examine the effects of packing density. This involves more elaborate preparation, and the change in vesicle size often results in factors, other than intrinsic binding considerations, that influence the dynamics of the protein-membrane interaction. The protein-vesicle collisional dynamics depend on vesicle size, and this can strongly influence the association rate constant (Abbott and Nelsestuen, 1987). Strict control of the lipid packing density is also not possible with the use of planar lipid bilayers. It is also well known that the use of PC/PS vesicles is complicated by vesicle fusion in the presence of Ca^{2+}, especially at higher PS contents. Such limitations are not encountered with the use of monolayers.

The origin of $\Delta\pi$ due to the adsorption of prothrombin at PC/PS monolayers

The interaction of prothrombin with POPS-containing LE monolayers resulted in an increase in π. If $\Delta\pi$ is the result of a hydrophobic interaction, then the extent of $\Delta\pi$ depends on the size of the penetrating species. A large $\Delta\pi$ is not expected for a peripheral protein that adsorbs without disrupting membrane properties. The relatively small $\Delta\pi$ values that have been observed here and elsewhere (Mayer et al., 1983a,b) for the adsorption of Gla domain proteins to monolayers are indicative of a small penetrating species.

In a recent study (Evans and Nelsestuen, 1996) it was suggested that the equilibrium surface pressure (π_e) of a protein film is related to the ability of the protein to penetrate phospholipid monolayers, and that the relatively low π_e of prothrombin fragment 1 (i.e., 9 mN/m) reflects low amphipathic structure (Evans and Nelsestuen, 1996). Although this may be true to a limited extent for the interaction between proteins and monolayers below π_e, it should not be related at all to the ability of small hydrophobic protein segments to penetrate the monolayer. Furthermore, π_e is affected by other factors besides amphipathicity, including the isoelectric point and pH.

If $\Delta\pi$ does not result from an insertion of hydrophobic segments, then it must be based in electrostatic interactions that rearrange the phospholipid headgroups. This was suggested by Mayer et al. (1983a). It can be speculated that there is competition for PS from Gla residues and that a Ca^{2+}-mediated PS-Gla complex simply occupies more area at the interface than a Ca^{2+}-mediated PS-PS complex. This may explain the sensitivity of $dc_s/d\pi$ to the PS content of LE (POPC/POPS) monolayers. If $\Delta\pi$ were entirely the result of a hydrophobic insertion, then $dc_s/d\pi$ would be

expected to be invariant with PS content. The adsorbing, penetrating species essentially results in a reduction in available area to the lipid and should occupy the same area in the monolayer, regardless of PS content. We have indicated that the slope of the π-A curve of 2:1 and 1:2 (m:m) POPC/POPS monolayers is invariant with PS content. Therefore, the penetrating species, which occupies a specified area in the monolayer, should produce a similar rise in pressure, regardless of PS content. Because $dc_s/d\pi$ is higher at lower PS contents, electrostatic interactions, such as the Ca^{2+}-mediated bridging interaction described above, may in fact contribute to the rise in pressure from the adsorption of prothrombin. The extent of this contribution appears to larger at higher PS contents. Nevertheless, because we observed a weak interaction between prothrombin and LC (DPPC/DPPS) monolayers, an electrostatic interaction appears to be a weak driving force for adsorption. The LC (DPPC/DPPS) monolayers possess a higher charge density at a given π_i than LE (POPC/POPS) monolayers. We have clearly indicated that, at a given π_i, increasing the PS mole ratio from 2:1 to 1:2 (m:m) POPC/POPS dramatically increases the affinity of prothrombin for LE monolayers. In addition, an increase in π_i of 2:1 (m:m) POPC/POPS LE monolayers produces a higher charge density of the monolayer, but this produced a lower affinity of prothrombin for the monolayer. Therefore, it is possible that hydrophobic interactions, which require a penetration of the monolayer, in addition to the electrostatic (Ca^{2+}-bridging) interactions, are a driving force for adsorption of prothrombin to the monolayers. A higher packing density clearly lowers the affinity of prothrombin for the monolayer.

One important factor that could limit the prothrombin-membrane association at higher packing densities is lipid diffusion. The lipid diffusion coefficient in LE monolayers decreases logarithmically with increasing film pressure (Li, 1995). This could affect either cooperative or noncooperative interactions. A higher lipid diffusion coefficient and higher PS mole fraction should increase the probability of a successful search for PS-binding sites when prothrombin encounters the surface by diffusion from the bulk phase. During the encounter time, slower PS diffusion to form the PS-Gla complex will lower the association rate constant. The dissociation rate constant can also be affected by lipid diffusion. Once the protein is released from its adsorption site, it remains at the interface as an encounter complex that must search the monolayer for another adsorption site or diffuse away from the interface and into the bulk solution.

We have shown that at similar packing densities, the K_d that characterizes the prothrombin interaction with 1:2 (m:m) POPC/POPS LE monolayers is an order of magnitude smaller than that with 2:1 (m:m) POPC/POPS LE monolayers. We have also shown that unlike 2:1 (m:m) POPC/POPS LE monolayers, the affinity of prothrombin for 1:2 (m:m) POPC/POPS LE monolayers is relatively insensitive to π_i. These results clearly demonstrate the existence of the electrostatic component of the adsorption. Although lipid diffusion could limit the electrostatic interaction, there is currently no solid evidence in support of this proposal.

The low affinity of prothrombin for DPPC/DPPS LC monolayers can be explained by a limitation on the hydrophobic interaction due to the high packing density. The insertion of a hydrophobic peptide fragment requires a defect in the ordered arrangement of the condensed lipid. The creation of such defects is entropically unfavorable (Peschke and Mohwald, 1987). On the other hand, the rigidity of the LC film and its influence on lipid mobility, as well as the arrangement of headgroups, may also influence the electrostatic interactions.

The lack of prothrombin adsorption to condensed lipid films has also been observed in a previous study. In this case, ellipsometry has been employed to quantify the interaction between prothrombin and PS multilayers consisting of either saturated dimyristoyl- or monounsaturated dioleoyl-PS (Cuypers et al., 1983). To obtain maximum adsorption of prothrombin to saturated lipid layers, the layers were heated for several minutes above the corresponding T_m. Heating was not a requirement when the unsaturated lipid was used, and maximum adsorption was observed at room temperature, which is above the T_m of the film. These investigators also noted that because the negative charge of the saturated lipid film was higher than that of the unsaturated film, other factors besides the negative charge were responsible for the prothrombin-PS interaction.

The use of vesicles to compare saturated and unsaturated lipids is apparently plagued by vesicle fusion. As pointed out previously (Higgins et al., 1985), it was not possible to calculate reliable K_d values describing the interaction between prothrombin and 5:1 (m:m) DMPC/DMPS vesicles, because of aggregation of the vesicles in the presence of Ca^{2+}. However, the authors did observe an apparent lack of detectable binding, or very little interaction, between prothrombin and the saturated lipid vesicles. Nonetheless, this did not have a large influence on the activation of prothrombin by the prothrombinase complex assembled on the vesicle surface.

A model of the adsorption that includes a hydrophobic insertion explains the weaker affinity of prothrombin for 2:1 (m:m) POPC/POPS LE monolayers at higher packing density and the weaker adsorption of prothrombin to 1:2 (m:m) DPPC/DPPS LC, relative to 1:2 (m:m) POPC/POPS LE monolayers. Therefore, if lipid diffusion does not influence the adsorption of prothrombin to monolayers, then a hydrophobic insertion is a necessity for adsorption. The role of the electrostatic bridging interaction may be similar to the electrostatic interaction involved in the adsorption of PBN. We determined that a hydrophobic insertion was a requirement for the adsorption of PBN. However, the hydrophobic interaction was not strong enough to overcome the affinity of the quaternary ammonium ion of PBN for the bulk solution. Otherwise, PBN would have adsorbed to POPC monolayers. On the other hand, when attractive forces between PBN and the phospholipid headgroups are operative, there is significant adsorption of PBN to LE monolayers. Because

adsorption of PBN is inhibited by the high packing density of LC monolayers, the electrostatic interaction, in itself, cannot produce significant adsorption of PBN. Such a mechanism could be operative with respect to adsorption of prothrombin. If so, then the Ca^{2+}-mediated bridging interaction is too weak to produce significant adsorption of prothrombin. This would explain why prothrombin does not readily adsorb to the 1:2 (m:m) DPPC/DPPS LC monolayer, but this is the case only if lipid diffusion is not limiting. Little is known about the influence of lipid diffusion, degree of lipid condensation, and lipid packing density on the electrostatic bridging interaction.

CONCLUSIONS

It is important to recognize that the use of phospholipid monolayers possesses certain advantages over the use of vesicles in describing the prothrombin-membrane interaction. The design developed here provides a simple means by which to measure the adsorbed protein concentration. This is particularly valuable for gauging whether protein adsorption occurs without a rise in π. Other monolayer approaches require radiolabeled protein and/or more costly and sophisticated measurement techniques. It is also important to recognize that a hydrophobic interaction appears to be a driving force for the adsorption of prothrombin (and likely other Gla-containing coagulation proteins) to membranes. In an attempt to increase our knowledge of this interaction, we are employing the approach developed here to examine interactions with spread monolayers of other vitamin K-dependent proteins and strategically designed recombinant mutants derived from these proteins.

This work was supported by grant HL-19982 from the National Institutes of Health and the Kleiderer-Pezold Family endowed professorship (to FJC). The authors also thank the Notre Dame Radiation Research Laboratory for technical assistance and for providing the electrobalance and Langmuir trough.

REFERENCES

Abbott, A. J., and G. L. Nelsestuen. 1987. Association of a protein with membrane vesicles at the collisional limit: studies with blood coagulation factor Va light chain also suggest major differences between small and large unilamellar vesicles. *Biochemistry.* 26:7994–8003.

Atkins, J. S., and P. R. Ganz. 1992. The association of human coagulation factor VIII, factor IXa and factor X with phospholipid vesicles involves both electrostatic and hydrophobic interactions. *Mol. Cell. Biochem.* 112:61–71.

Banner, D. W., A. D'Arcy, C. Chene, F. K. Winkler, A. Guha, W. H. Konigsberg, Y. Nemerson, and D. Kirchofer. 1996. The crystal structure of the complex of blood coagulation factor VII with soluble tissue factor. *Nature.* 340:41–46.

Christiansen, W. T., L. R. Jalbert, R. M. Robertson, A. Jhingan, M. Prorok, and F. J. Castellino. 1995. Hydrophobic amino acid residues of human anticoagulation protein C that contribute to its functional binding to phospholipid vesicles. *Biochemistry.* 34:10376–10382.

Cuypers, P. A., J. W. Corsel, M. P. Janssen, J. M. M. Kop, W. T. Hermens, and H. C. Hemker. 1983. The adsorption of prothrombin to phosphatidylserine multilayers quantitated by ellipsometry. *J. Biol. Chem.* 258: 2426–2431.

Demel, R. A., W. S. M. Geurts Van Kessel, R. F. A. Zwaal, B. Roefofsen, and L. M. Van Deenen. 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. *Biochim. Biophys. Acta.* 406:97–107.

Demel, R. A., F. Paltauf, and H. Hauser. 1987. Monolayer characteristics and thermal behavior of natural and synthetic phosphatidylserines. *Biochemistry.* 26:8659–8665.

Ellison, E. H., and J. K. Thomas. 1996. Photophysical studies of ultrathin films: characterization of PS and PMMA on fused quartz by fluorescence spectroscopy of pyrene and (4-(1-pyrenyl)butyl)trimethylammonium bromide. *Langmuir.* 12:1870–1878.

Evans, T. C., and G. L. Nelsestuen. 1996. Importance of cis-proline 22 in the membrane-binding conformation of bovine prothrombin. *Biochemistry.* 35:8210–8215.

Feigenson, G. W. 1986. On the nature of calcium ion binding between phosphatidylserine lamellae. *Biochemistry.* 25:5819–5825.

Gaines, G. L. 1977. On the use of filter paper Wilhemly plates with insoluble monolayers. *J. Colloid Surf. Sci.* 62:191–192.

Higgins, D. L., P. J. Callahan, F. G. Prendergast, M. E. Nesheim, and K. G. Mann. 1985. Lipid mobility in the assembly and expression of the activity of the prothrombinase complex. *J. Biol. Chem.* 260:3604–3612.

Israelachvili, J. N., D. J. Mitchell, and B. W. Ninham. 1977. Theory of self-assembly of lipid bilayers and vesicles. *Biochim. Biophys. Acta.* 470:185–200.

Jahnig, F. 1996. What is the surface tension of a lipid bilayer membrane? *Biophys. J.* 71:1348–1349.

Jalbert, L. R., J. C. Y. Chan, W. T. Christiansen, and F. J. Castellino. 1996. The hydrophobic nature of residue-5 of human protein C is a major determinant of its functional interactions with acidic phospholipid vesicles. *Biochemistry.* 35:7093–7099.

Kop, J. M. M., P. A. Cuypers, T. Lindhout, H. C. Hemker, and W. T. Hermens. 1984. The adsorption of prothrombin to phospholipid monolayers quantitated by ellipsometry. *J. Biol. Chem.* 259:13993–13998.

Lecompte, M. F., and C. Dode. 1992. Embedding of prethrombin domains of human prothrombin into phospholipid membranes. *Bioelectrochem. Bioenerg.* 29:149–157.

Li, L. 1995. Photoinduced electron transfer between lipid-functionalized pyrene and dipyridinium derivatives in Langmuir films and vesicles. Ph.D. thesis. University of Notre Dame, Notre Dame, IN.

Lu, Y. F., and G. L. Nelsestuen. 1996. Dynamic features of prothrombin interaction with phospholipid vesicles of different size and composition: implications for protein-membrane contact. *Biochemistry.* 35: 8193–8200.

MacRitchie, F. 1990. Chemistry at Interfaces. Academic Press, San Diego, CA.

Mann, K. G., M. E. Neshiem, W. R. Church, P. Haley, and S. Krishnaswamy. 1990. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood.* 76:1–16.

Mayer, L. D., G. L. Nelsestuen, and H. L. Brockman. 1983a. Prothrombin association with phospholipid monolayers. *Biochemistry.* 22:316–321.

Mayer, L. D., M. L. Pusey, M. A. Griep, and G. L. Nelsestuen. 1983b. Association of blood coagulation factors V and X with phospholipid monolayers. *Biochemistry.* 22:6226–6232.

Mingotaud, A. F., C. Mingotaud, and L. K. Patterson. 1996. Handbook of Monolayers, Vol. 1. Academic Press, San Diego, CA.

Nelsestuen, G. L., and T. K. Lim. 1977. Equilibria involved in prothrombin- and blood clotting factor X-membrane binding. *Biochemistry.* 16: 4164–4171.

Peschke, J., and H. Mohwald. 1987. Cytochrome c interaction with phospholipid monolayers and vesicles. *Colloids Surfaces.* 27:305–323.

Rodgers, G. M., and M. A. Shuman. 1983. Prothrombin is activated on vascular endothelial cells by factor Xa and calcium. *Proc. Natl. Acad. Sci. USA.* 80:7001–7005.

Soriano-Garcia, M., K. Padmanabhan, A. M. deVos, and A. Tulinsky. 1992. The Ca^{2+} ion and membrane binding structure of the gla-domain of Ca-prothrombin fragment 1. *Biochemistry.* 21:2554–2566.

Swords, N. A., and K. G. Mann. 1993. The assembly of the prothrombinase complex on adherent platelets. *Atheroscler. Thromb.* 13:1602–1612.

Tendian, S. W., B. R. Lentz, and N. L. Thompson. 1991. Evidence from total internal reflection fluorescence microscopy for calcium-independent binding of prothrombin to negatively charged planar phospholipid membranes. *Biochemistry.* 30:10991–10999.

Tracy, P. B., L. L. Eide, and K. G. Mann. 1983. Human prothrombinase complex assembly on isolated peripheral blood cell populations. *J. Biol. Chem.* 260:2119–2124.

Wei, G. J., V. A. Bloomfield, R. M. Resnick, and G. R. Nelsestuen. 1982. Kinetic and mechanistic analysis of prothrombin-membrane binding by stopped-flow light scattering. *Biochemistry.* 21:1949–1959.

Zhang, L., and F. J. Castellino. 1994. The binding energy of human coagulation protein C to acidic phospholipid vesicles contains a major contribution from leucine-5 in the gamma-carboxyglutamic acid domain. *J. Biol. Chem.* 269:3590–3595.