Adsorption of Vitamin K-Dependent Blood Coagulation Proteins To Spread Phospholipid Monolayers as Determined from Combined Measurements of the Surface Pressure and Surface Protein Concentration†

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ABSTRACT: Spread phospholipid monolayers are particularly useful as model membranes in that changes in surface pressure ($\Delta\pi$) can be monitored in response to protein adsorption to the monolayer, thus providing a unique manner of assessing protein−membrane contact. In the present study, spread monolayers below their collapse pressures have been utilized to evaluate Ca^{2+}-specific adsorption of several vitamin K-dependent coagulation proteins to monolayers that contain negatively charged phospholipid. From combined measurements of $\Delta\pi$ and Γ (the surface excess protein concentration), values of $d\Gamma/d\pi$ have been evaluated for different proteins with varying lipid composition of the monolayers. Using mixed, liquid-expanded monolayers at equivalent initial surface pressures (π_i) and which contain different amounts of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine, the $d\Gamma/d\pi$ of bovine prothrombin was shown to decrease monotonically with increasing protein affinity for the monolayer. For example, K_D values of 7, 20, and 60 nM produced $d\Gamma/d\pi$ values of 14, 17, and 21 nmol m^{-1} mN^{-1}, respectively. However, the trend in $d\Gamma/d\pi$ appears to originate from characteristics of the monolayer and not from those of the protein, since a much different adsorbate (i.e., a positively charged pyrene derivative) exhibited a similar trend in $d\Gamma/d\pi$ with monolayer composition. On the other hand, $d\Gamma/d\pi$ values of bovine prothrombin, human factor IX, human protein S, bovine protein C, and human protein C, determined using liquid-expanded phosphatidylserine monolayers, were essentially equivalent. Therefore, the five vitamin K-dependent proteins that were examined were equivalent in terms of the manner in which the γ-carboxyglutamic acid (Gla) domain of each protein perturbed the surface pressure. This study shows that Ca^{2+}-specific membrane contact sites in the Gla domain of the five proteins tested are similar despite the naturally occurring differences in the normal Gla domain sequence of these proteins.

The vitamin K-dependent coagulation plasma proteins possess from 9 to 12 residues of γ-carboxyglutamic acid (Gla) concentrated in an ~40 amino acid peptide sequence, that is, the Gla domain, which encompasses the NH_2-terminal region. In the presence of Ca^{2+} and negatively charged phospholipids, the Gla domain functions as the site of protein attachment to membranes. Adsorption of the Gla-containing coagulation proteins to membranes is critical to proper function of the blood coagulation system. Many reactions involving these proteins are highly enhanced at membrane surfaces (*1*).

One special feature of the Gla domain is the high degree of sequence homology found among the various Gla-containing coagulation proteins. However, the affinity of these proteins for equivalent model membranes can vary by as much as 2 orders of magnitude. A topic of current interest is whether these varied affinities are related to differences in the Gla domains of these proteins. For example, it is unclear whether proteins with a larger number of Gla residues make additional contacts with the membrane, or, more generally, if actual protein attachment to membranes is related to more subtle differences in the Gla domain sequences between proteins. This type of information is important in developing future strategies for understanding and/or mimicking protein-membrane contact.

In a previous contribution, a new and useful approach was developed to quantify the concentration of bovine prothrombin (bPt) adsorbed to liquid-expanded phospholipid monolayers containing phosphatidylcholine (PhC) and phosphatidylserine (PhS) (*2*). The methods employed simultaneous determinations of the increase in surface excess protein concentration (Γ), and the corresponding increase in surface pressure ($\Delta\pi$), following injection of prothrombin into the aqueous subphase of spread monolayers. Measurements of Γ were made possible by developing new methodology for evaluating continual depletion of the subphase protein concentration due to adsorption to monolayers.

In the current study, this approach was extended to clarify the source of variation in $d\Gamma/d\pi$ with monolayer composition. Additionally, $d\Gamma/d\pi$ was evaluated for several Gla-containing proteins exhibiting differences in K_D of more than 2 orders of magnitude, along with the use of $d\Gamma/d\pi$ as an indicator

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of protein-membrane contact. The results of these studies are summarized in the present communication.

MATERIALS AND METHODS

Materials. Stock solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. The concentration of phospholipid in the stock solutions was determined by weighing a known volume of lipid to the nearest 0.01 mg following evaporation of the solvent in vacuo. The standard buffer contained 10 mM Tris-HCl/100 mM NaCl/0.5 mM EDTA, pH 7.4. $CaCl_2$ was added to the buffer when required. High-purity water (TOC < 3 ppb) was obtained in-house from a Barnstead Easypure-UV system.

Plasma proteins including human protein S (hPS), bovine prothrombin (bPt), human factor IX (hfIX), and bovine protein C (bPC) were obtained from the Enzyme Research Corp. (South Bend, IN). The generation and purification of wild-type-recombinant human protein C (wtr-hPC) and the mutant, r-[L8Q]hPC, have been described previously (*3, 4*). Prior to use, all proteins were dialyzed against the standard buffer at 4 °C. The protein concentrations were determined by absorption spectroscopy utilizing $\epsilon_{280,1\%} = 1.44$, 0.95, 1.32, 1.45, 1.45, and 1.37 mL mg^{-1} cm^{-1} of bPt, hPS, hfIX, wtr-PC, r-[L8Q]hPC, and bPC, respectively. PBN [(4-(1-pyrenyl)butyl)trimethylammonium bromide] was purchased from Molecular Probes, Inc. (Eugene, OR).

Apparatus. The approach developed to evaluate protein adsorption to spread phospholipid monolayers has been previously described (*2*). This involves the use of a 32-mL quartz trough filled with buffer solution. The trough was housed in a plexiglass chamber to control the atmosphere. One drop of lipid solution was carefully spread at the air−water interface, and following stabilization of π, the protein was injected into the subphase. With stirring, π and the subphase fluorescence intensity (I_f) were continuously and simultaneously monitored as the protein adsorbed to the monolayer. The Wilhemly technique was employed to monitor π, using filter paper as the Wilhemly plate (*2, 5*).

Measurements of the subphase fluorescence intensity were made using an SLM-8000 spectrofluorometer, with a 450-W xenon arc lamp. To monitor fluctuations in lamp energy, a portion of the excitation beam was reflected, using a quartz slide, into a reference PMT. Hamamatsu R928 photomultiplier tubes were used to monitor emission and reference signals. An air-driven stirrer, located external to the plexiglass chamber, was used to stir the subphase at a constant rate of 90 rpm during the course of all measurements. The use of an air-driven stirrer was necessary because electric-powered stirrers produced heat which interfered with the temperature control of the system.

Fluorescence and electrobalance readings were collected simultaneously at a rate of 0.1 Hz. The output from the electrobalance was analogue-filtered and subsequently digitized using a DAS-801 (Omega) data acquisition board controlled by standard computer software.

Monolayers. Monolayers were prepared by carefully spreading 5−6 μL of lipid solution ($c_{lipid} \approx 1.1$ mM) on the buffer surface. After spreading, the surface pressure stabilized within 30 min. The spreading solvent was 10:90 (v/v) EtOH/hexane. All monolayer compositions are expressed in this report on a mole ratio basis. The basis for choosing the synthetic lipids utilized has been described (*2*).

The chamber was purged with a flowing stream of compressed purified air (in-house laboratory air supply) which was bubbled through water immediately prior to entering the chamber. This atmosphere was sufficient to maintain a stable pressure reading of all monolayers tested. When unsaturated hydrocarbon monolayers were exposed to room air, the pressure reading increased rapidly with time. This was the result of ozone, which reacts with unsaturated hydrocarbon monolayers (*6*), and which is not present in the in-house air supply.

Pretreatment of the Quartz Trough. Prior to use, the quartz trough was scrubbed using a soft oil-free tissue and microcleaner, and subsequently rinsed with copious amounts of ethanol and water. When the adsorption properties of PBN to monolayers were to be tested, the previously cleaned trough was filled once with 10^{-6} M PBN, followed by 0.1 M NaCl, then thoroughly rinsed with water. This procedure was necessary to prevent PBN from adsorbing to the quartz surface and thus depleting it erroneously from the subphase concentration.

Table 1: Variation of K_D and $d\Gamma/d\pi$ of Bovine Prothrombin with Lipid Composition of Spread Monolayers

lipid composition of monolayer ($\pi_i = 30 \pm 1$ mN/m)	K_D of bPt (nM)	$d\Gamma/d\pi$ of bPt[a] (nmol m^{-1} mN^{-1})	$d\Gamma/d\pi$ of PBN[b] (nmol m^{-1} mN^{-1})
POPS	nd[c]	11	nd
1:2 (m/m)			
POPC/POPS	7[d]	14	123 (18)
1:1:1 (m/m/m)			
POPC/POPE/POPS	20	17	nd
2:1 (m/m)			
POPC/POPS	60[d]	21	174 (23)

[a] Values of $d\Gamma/d\pi$ were obtained from plots of Γ versus $\Delta\pi$. The subphase contained 10 mM $CaCl_2$. [b] Ca^{2+} was not included in the buffer subphase. [c] Not determined. [d] Data from ref 2.

RESULTS

Variation in $d\Gamma/d\pi$ with Lipid Composition of Monolayers. In Table 1 values are provided of $d\Gamma/d\pi$ for bPt at various lipid compositions of spread phospholipid monolayers. These data were obtained at $\pi_i = 30 \pm 1$ mN/m, well below the collapse pressure of mixed POPC/POPS monolayers, which range from 42 to 45 mN/m. Also, at $\pi_i = 30$ mN/m, the packing density has been shown to be similar to natural membranes (*7*). In the present study, careful measurements of the background decrease in fluorescence intensity, and subsequent correction for this effect, have produced slightly lower values of $d\Gamma/d\pi$ relative to previous results. However, the more general result, that of the decrease of $d\Gamma/d\pi$ with higher POPS content, was the same.

Also included in Table 1 are values of K_D at each monolayer composition. The K_D values were evaluated according to the Langmuir relation from measurements of $1/\Delta\pi$ versus $1/c_b$, as previously described (*2*). The influence of substituting POPE for POPC on the affinity of bPt for monolayers has been evaluated, where 1:1:1 POPC/POPE/POPS monolayers were tested for binding efficacy of this

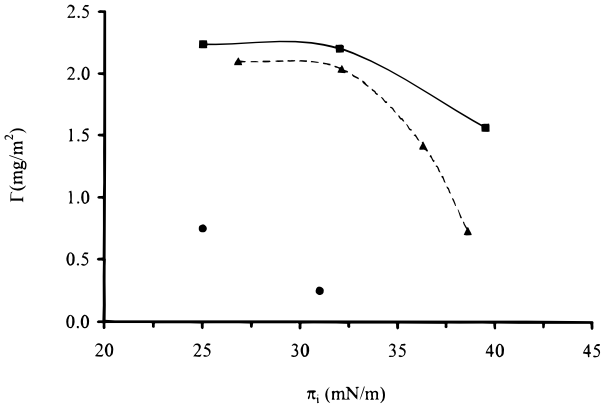

FIGURE 1: The dependence of Γ at equilibrium on π_i and the POPE content of monolayers, when c_b (bPt) = 0.040 μM: (■), 1:1:1 POPC/POPS/POPE monolayers; (▲), 2:1 POPC/POPS monolayers; (●), POPE monolayers. The buffer contained 10 mM Tris-HCl/100 mM NaCl/10 mM $CaCl_2$, pH 7.4, 23 °C.

protein. In this case, 50% of the POPC molecules in a 2:1 POPC/POPS monolayer have been substituted with POPE. This produced a significant decrease in the K_D. To further illustrate this effect, values of Γ were evaluated at $c_b = 0.040$ μM bPt and at various π_i of monolayers. The data obtained are illustrated in Figure 1, where it is evident that the increase in affinity due to POPE substitution is more prevalent at higher packing density. The plots of Figure 1 also show that pure POPE monolayers at low π_i can support a low level of binding of bPt. This result is somewhat surprising, considering that POPE is zwitterionic, as is POPC, and Ca^{2+}-specific binding of bPt to pure POPC monolayers has not been observed. In the absence of Ca^{2+}, binding of bPt to POPE monolayers was not detected. The increased affinity of bPt for POPE-containing monolayers was not the result of direct binding to POPE, since bPt did not interact with 1:2 POPC/POPE monolayers at $\pi_i > 30$ mN/m, at $c_b = 0.040$ μM. The 1:2 POPC/POPE monolayer does not contain POPS, but is at an identical POPC mole ratio relative to 1:1:1 POPC/POPE/POPS monolayers.

Values of Γ at saturation binding (Γ_{sat}) were also determined using 1:1:1 POPC/POPE/POPS monolayers and compared to that same binding at 1:2 POPC/POPS monolayers. An accurate approach to determining Γ_{sat} is to measure $\Delta\pi_{sat}$ by injecting protein into the subphase to produce c_b at least 10× in excess of K_D. The value of Γ_{sat} is then calculated according to $\Gamma_{sat} = (\Delta\pi_{sat})(d\Gamma/d\pi)$. Figure 2A illustrates two plots of $\Delta\pi$ versus time following the injection of excess prothrombin into the subphase of either 1:2 POPC/POPS or 1:1:1 POPC/POPE/POPS monolayer at $\pi_i = 32$ mN/m. During the latter stages of adsorption, a significant difference in $\Delta\pi$ was seen. Leveling off of π was not observed, even after 4 h. This is most likely the result of nonspecific adsorption (*2*), which appears to be very similar in both monolayers. Figure 2B illustrates a plot of Γ versus time, where values of $d\Gamma/d\pi$ in Table 1 were used to calculate Γ. It is apparent that Γ_{sat} is invariant between the two monolayers. In a previous study, it was indicated that Γ_{sat} was invariant with POPS content and π_i (in the region $\pi_i = 25-39$ mN/m), when 2:1 POPC/POPS and 1:2 POPC/POPS monolayers were compared (*2*). In the present study, comparing different monolayers, it is shown once again that Γ_{sat} is independent of POPS content, as well as the presence of POPE.

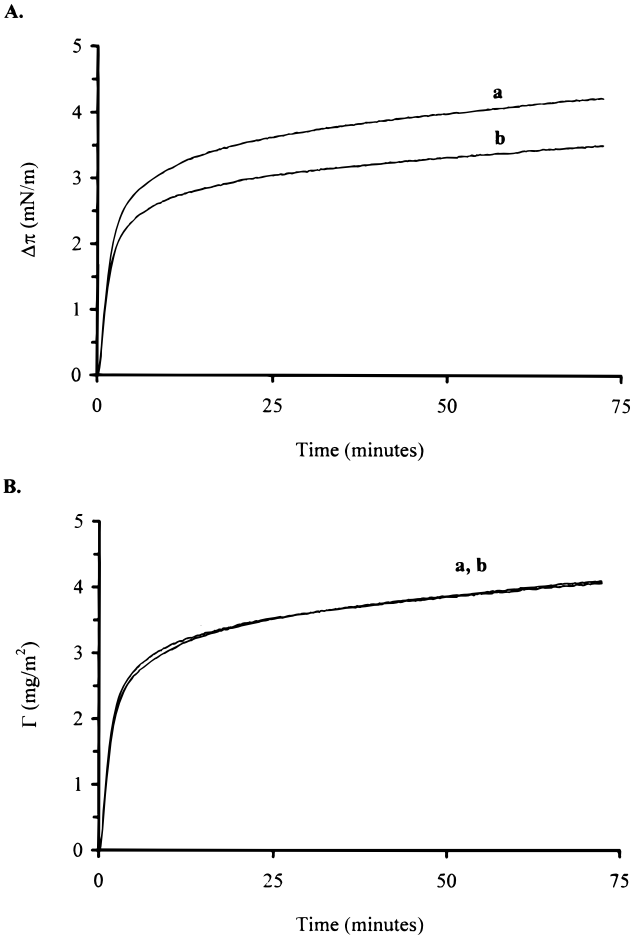

FIGURE 2: Profiles of $\Delta\pi$ and Γ versus time following the injection of excess bPt into the subphase of spread monolayers. (A) $\Delta\pi$ versus time when c_b (bPt) = 0.65 μM: (a), 1:2 POPC/POPS monolayer at $\pi_i = 32$ mN/m; (b), 1:1:1 POPC/POPS/POPE monolayer at $\pi_i = 32$ mN/m; T = 23.0 ± 0.3 °C. (B) Γ versus time, under the conditions described in A. The buffer was composed of 10 mM Tris-HCl/100 mM NaCl/10 mM $CaCl_2$, pH 7.4, 23 °C.

From the data in Figure 2B, it is seen that the area occupied by prothrombin at Γ_{sat} is estimated to be 3000−4000 Å²/molecule. This corresponds to a circular diameter of 60−70 Å. The values of the length and diameter of the ellipsoid of revolution representing prothrombin were determined to be 119 and 34 Å, respectively (*8*). Thus, bPt is apparently in a tilted orientation at the interface and close packing is what determines the number of binding sites at these monolayer surfaces where excess PhS is present.

As indicated in Table 1, $d\Gamma/d\pi$ values of bPt vary with lipid composition of the monolayer and become larger with increasing K_D. To more adequately investigate the source of these differences, the manner in which $d\Gamma/d\pi$ varies for the fluorescent pyrene derivative, PBN, has been evaluated. In a previous study, it was observed that adsorption of PBN to a 1:2 POPC/POPS monolayer produced significant depletion of the subphase PBN concentration and a measurable $\Delta\pi$ (*2*). In the present study, $d\Gamma/d\pi$ of PBN has been evaluated and it was found that this parameter varies with lipid composition in much the same way as that of bPt. Figure 3 illustrates representative data used to determine $d\Gamma/d\pi$ of PBN. Higher PhS content in the monolayer produced larger changes in Γ and π. The values of $d\Gamma/d\pi$ for PBN listed in Table 1 indicate that $\Delta\pi$ is 1.4-fold less sensitive

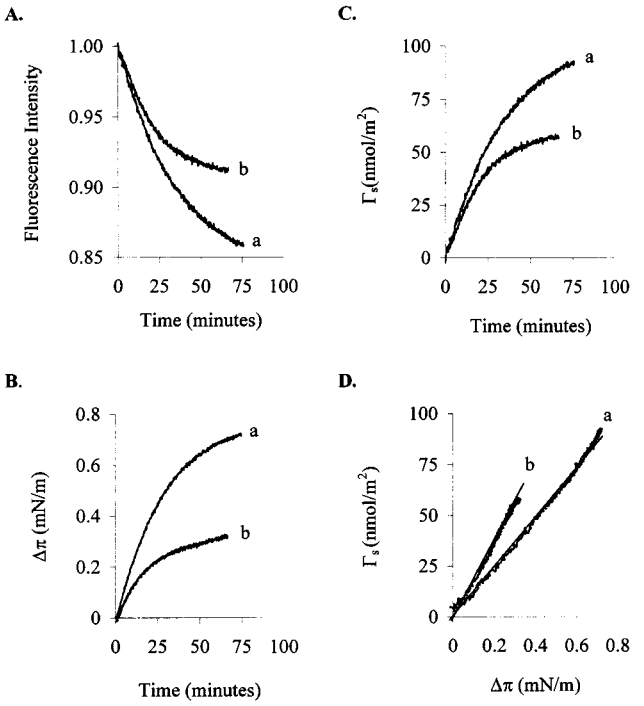

FIGURE 3: Influence of the POPS content in monolayers on $d\Gamma/d\pi$ of PBN. Representative plots of the decrease in subphase fluorescence intensity (A), the increase in π (B), and the increase in Γ (C) following the injection of PBN into the subphase of a 1:2 POPC/POPS monolayer at $\pi_i = 30$ mN/m [curve a in graphs A, B, C, and D] and a 2:1 POPC/POPS monolayer at $\pi_i = 30$ mN/m (curve b in graphs A, B, C, and D). c_b (PBN) = 0.040 μM. No decrease in the subphase fluorescence intensity or increase in π was observed when a monolayer was not present at the interface. (D) Plot of Γ versus $\Delta\pi$ from the data in graphs B and C, which indicates lower sensitivity of $\Delta\pi$ to Γ in 2:1 POPC/POPS monolayers. All data were obtained using a buffer containing 10 mM Tris-HCl/100 mM NaCl/0.5 mM EDTA, pH 7.4, 23 °C. Ca^{2+} was omitted from this buffer so that PBN would not compete with Ca^{2+} for the negative charge of the monolayer.

as an indicator of PBN adsorption to 2:1 POPC/POPS monolayers, relative to 1:2 POPC/POPS monolayers. This is very close to the ratio of $d\Gamma/d\pi$ observed for bPt, which was 1.5. However, each adsorbed PBN molecule produces roughly an order of magnitude smaller $\Delta\pi$ than each adsorbed prothrombin molecule.

Variation in $d\Gamma/d\pi$ between Different Gla-Containing Proteins. The nature of the variation in $d\Gamma/d\pi$ between Gla-containing proteins with large differences in affinity for membranes has also been evaluated. For the purposes of comparing $d\Gamma/d\pi$ of these proteins, we were restricted to using pure POPS monolayers because these proteins exhibited the highest affinity for POPS monolayers, and this high affinity was necessary to observe binding and subphase depletion of the weaker-affinity proteins. Values of $d\Gamma/d\pi$ of five such proteins spanning a K_D range of more than 2 orders of magnitude are listed in Table 2. In determining values of Γ, the background drop in subphase fluorescence intensity was carefully evaluated for each protein. Our approach, utilizing spread liquid-condensed ($\pi_i > 30$ mN/m) DPPC monolayers to evaluate the background drop in fluorescence intensity, has been described (*2*).

All values of $d\Gamma/d\pi$ were evaluated at $\pi_i = 30 \pm 1$ mN/m. A phase transition occurs for POPS monolayers in the region $\pi_i = 35$ mN/m (*9*). When $d\Gamma/d\pi$ was evaluated in this latter region, much higher values were observed. For example, at $\pi_i = 35$ mN/m, $d\Gamma/d\pi$ values of bPt and hPC were 21 and 16 nmol m^{-1} mN^{-1}, respectively. This was expected because the slope of the π area curve of POPS above 35 mN/m is less steep. Therefore, $\Delta\pi$ becomes smaller for a given Γ, and $d\Gamma/d\pi$ increases.

Table 2: $d\Gamma/d\pi$ of Gla-Containing Proteins with Known Differences in Affinity for Model Membranes

protein	K_D[a] (nM)	$d\Gamma/d\pi$[b] (nmol m^{-1} mN^{-1})
human protein S	51	11.4 (1.4)
bovine prothrombin	110	10.9 (0.8)
human factor IX	1000	8.9 (0.7)
human protein C	1500	8.4 (0.5)
bovine protein C	>15 000	12.7 (1.4)

[a] Values from Table 2 in ref 10. [b] Determined by using POPS monolayers at $\pi_i = 30 \pm 1$ mN/m. $[Ca^{2+}] = 10$ mM. Errors reported are at a 95% confidence interval.

The values of K_D listed in Table 2 are those taken from a recent study which summarized protein-bilayer vesicle binding data (*10*). It was indicated in a previous study that spread monolayers produce similar K_D values of bPt relative to bilayer vesicles of the same PL composition (*2*). To further illustrate that spread monolayers are similar to these vesicles, the affinity of hPC for spread monolayers has been determined. The K_D value for binding of hPC to a 1:2 POPC/POPS monolayer at $\pi_i = 32$ mN/m was 80 nM, which is an order of magnitude higher than that of bPt (i.e., $K_D = 7$ nM in Table 1). The K_D values in Table 2 also indicate an approximate order of magnitude difference in affinity when hPC and bPt are compared, although these values were collected at lower relative PhS content in vesicles. Under these latter conditions, for example, 2:1 POPC/POPS, we found that the affinity of hPC for monolayers at $\pi_i > 25$ mN/m was very weak. This low affinity restricted the analysis of K_D, since unduly large amounts of hPC were required for its measurement and subphase depletion measurements were unreliable. However, the affinity of bPt for 2:1 POPC/POPS monolayers was an order of magnitude lower relative to 1:2 POPC/POPS monolayers (see Table 1). This may be a reasonable approximation for the difference in affinity of hPC for 2:1 and 1:2 POPC/POPS monolayers.

The data in Table 2 indicate that values of $d\Gamma/d\pi$ of hfIX and hPC are significantly lower than the values for the other proteins tested. This is mainly due to the influence of nonspecific adsorption on $\Delta\pi$. Figure 4 illustrates the tendency for nonspecific adsorption, as evaluated from the analysis of $\Delta\pi$ of pure POPC monolayers at $\pi_i = 20$ mN/m. Ca^{2+}-specific adsorption to POPC monolayers does not occur to any appreciable extent. It is apparent that hfIX and hPC produce much larger values of $\Delta\pi$ in POPC monolayers. This indicates greater tendency for nonspecific adsorption relative to the other proteins tested. None of the systems in Figure 4 exhibited measurable subphase depletion. This indicates that the areas occupied in the film by nonspecifically adsorbed protein are much higher than that of specifically adsorbed protein.

The data in Figure 4 may qualitatively account for the differences in $d\Gamma/d\pi$ in Table 2. For hfIX and hPC, the $\Delta\pi$ evaluated using POPS monolayers could include a significant contribution from nonspecific adsorption. However, to

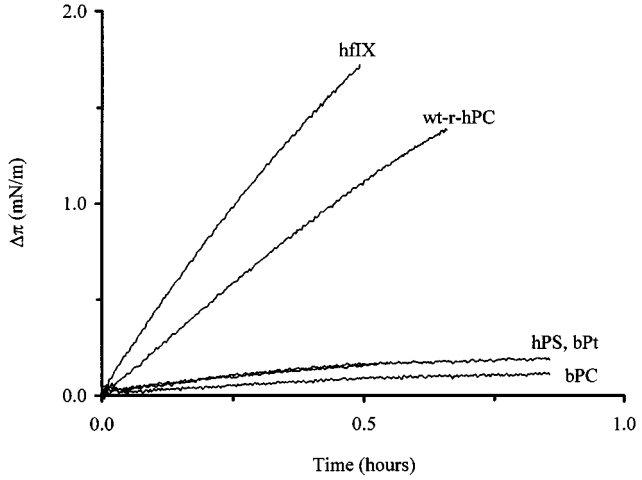

FIGURE 4: The $\Delta\pi$ values of POPC monolayers resulting from nonspecific adsorption of various Gla-containing proteins. The conditions were $\pi_i = 20$ mN/m and c_b (protein) $= 0.040$ μM. The buffer contained 10 mM Tris-HCl/100 mM NaCl/10 mM $CaCl_2$, pH 7.4, 23 °C.

compare hfIX and hPC with the other proteins, $\Delta\pi$ due to Ca^{2+}-specific adsorption must be quantitatively evaluated. To do so, $\Delta\pi$ of a mutant r-PC, r-[L8Q]hPC, has been evaluated, in which the affinity of the protein for membranes has been significantly lowered, such that Ca^{2+}-specific adsorption is no longer observed. The data obtained are illustrated in Figure 5. This mutant hPC was shown in a previous study to exhibit very low affinity for PhC/PhS-containing vesicles (*4*). In testing depletion of subphase r-[L8Q]hPC concentration below POPS monolayers (Figure 5B), no significant difference was found relative to the background measurement (taken as that below DPPC monolayers). This was not the case for wtr-hPC which exhibited a significant drop in I_f (Figure 5B) and a much larger $\Delta\pi$ (Figure 5A) relative to r-[L8Q]hPC. The $\Delta\pi$ of this mutant (Figure 5A) is a reasonable approximation of $\Delta\pi$ due to nonspecific adsorption of wtr-hPC. Upon subtraction of this $\Delta\pi$ from that same value of wtr-hPC, an estimate of the Ca^{2+}-specific component of $\Delta\pi$ was obtained. Following this conversion, the value of $d\Gamma/d\pi$ for hPC increased from 8.4 to 11.4 nmol m^{-1} mN^{-1}. The latter value is within the range of values determined for the three proteins not exhibiting significant nonspecific adsorption. A similar situation is also likely for hfIX.

It must be recognized that the correction to $d\Gamma/d\pi$ of hPC may be subject to some degree of error. Ca^{2+}-specific adsorption of wtr-hPC produces a surface excess protein concentration at the lipid−water interface. This surface excess may increase the rate of nonspecific adsorption. No surface excess of r-[L8Q]hPC was observed. Thus, $\Delta\pi$ estimated to be due to nonspecific adsorption may underestimate the true value. Thus, the corrected $d\Gamma/d\pi$ value of hPC may be slightly lower than the true value. However, nonspecific adsorption of specifically bound protein may be inhibited somewhat from the usual case in that the Ca^{2+}-specific interaction may restrict the surface orientation required for nonspecific adsorption.

DISCUSSION

In this study, precise measurements of $d\Gamma/d\pi$ have been made under various conditions. A notable result was that

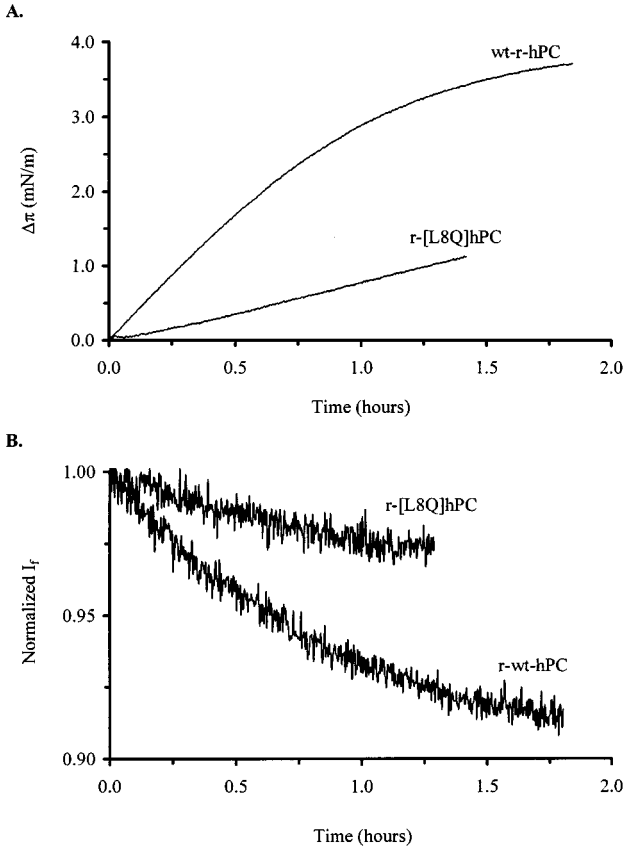

FIGURE 5: Adsorption of wtr-hPC and r-[L8Q]hPC to POPS monolayers. (A) $\Delta\pi$ of POPS monolayer following the injection of protein into the subphase. (B) Decrease in subphase fluorescence intensity corresponding to the data in A. The conditions were $\pi_i =$ 30 mN/m and c_b (protein) $= 0.040$ μM, in 10 mM Tris-HCl/100 mM NaCl/10 mM $CaCl_2$, pH 7.4, 23 °C.

$d\Gamma/d\pi$ of bPt and the fluorescent probe molecule PBN varied in a similar way with changes in monolayer composition. Thus, the trend in $d\Gamma/d\pi$ for bPt appears to originate from the intrinsic properties of the monolayer, and not from those of the protein, since this value was equivalent when two very different adsorbates were compared. The dependency of $d\Gamma/d\pi$ on the nature of the monolayer could result from a number of sources related to lipid composition, for example, modulations of interfacial water activity and lipid−lipid interactions. This is a difficult problem to resolve given the complexities at the lipid−water interface and the inadequacies of present theoretical models to describe such effects.

In evaluating $d\Gamma/d\pi$ of five different Vitamin K-dependent coagulation proteins, two of these, namely, hPC and hfIX, exhibited significantly lower values of $d\Gamma/d\pi$ relative to the other three (bPt, hPS, and bPC). This was explained by the possible influence of nonspecific adsorption on the measurement of $\Delta\pi$ due to Ca^{2+}-specific adsorption ($\Delta\pi_{Ca}$). A first-order correction of $\Delta\pi$, which accounted for nonspecific adsorption, produced a value of $d\Gamma/d\pi$ of hPC similar to the values for those proteins exhibiting lower tendency for nonspecific adsorption. Thus, it appears that $\Delta\pi_{Ca}$ (at a given Γ) is essentially invariant between this set of proteins which differ largely in their affinity for membranes. This is exemplified by the fact that bPt, hPS, and bPC represent the upper and lower ends of the range of K_D values tested, and all of these proteins exhibited low (and similar) tendencies

for nonspecific adsorption, as well as similar values of $d\Gamma/d\pi$.

The origin of $\Delta\pi_{Ca}$ cannot be resolved from this approach. It may result from the disruption of lipid headgroups via the Ca^{2+}-dependent bridging interactions, or possibly from the insertion of hydrophobic side chains into the hydrocarbon region of the monolayer (*11*). Either process can potentially produce an expansion of the monolayer. However, the Ca^{2+}-specific binding interaction with POPS monolayers produced similar values of $d\Gamma/d\pi$ for the five proteins tested. Therefore, Ca^{2+}-specific membrane contact sites in the Gla domain are similar for these proteins, at least in terms of the manner in which they perturb the surface pressure of POPS monolayers. Given that the variability in $d\Gamma/d\pi$ for bPt with monolayer composition did not originate from the protein, it seems likely that the invariability in $d\Gamma/d\pi$ between different vitamin K-dependent coagulation proteins should extend to other monolayer compositions.

Despite a similar contact mechanism, differences in the Gla domain sequences of the proteins could yet account for variation in protein affinity for membranes by modulating exposure of membrane contact sites, or the equilibrium distribution between binding and nonbinding conformers. However, recent investigations of chimeric hPC molecules indicated that the function of the Gla domain may be purely general. In two separate studies, the Gla domain and trailing helical stack of hPC were replaced by those of either hfIX (*12*) or hfVII (*13*). Both substitutions had little effect on protein-binding affinity and activity as compared to wtr-hPC. However, relative to hPC, hfVII exhibits an order of magnitude lower affinity for membranes. Thus, differences in protein affinity for membranes can originate, directly or indirectly, from regions other than the Gla domain.

One other result in this study requiring consideration is the effect of substituting PhE for PhC in mixed (PhC/PhS) monolayers on subsequent binding of bPt to monolayers. The results of this examination were obtained under very controlled conditions. The surface area and organization of the lipid assembly remain constant when PhE is substituted for PhC in monolayers. However, in bilayer systems such as vesicles, the inclusion of PhE can produce hexagonal structures as the content of bilayer-forming lipids decreases (*14*). In most cases, the effects of these irregular structures on protein binding and activity are not known. This was the situation in a recent study of PhE effects on activated protein C activity in vitro (*15*). Nevertheless, our results are in accord with previous studies of protein binding to bilayer systems, where there was not as much certainty about the size and organization of the PhE-containing lipid assembly (*16*−*18*). Therefore, the data obtained in the present investigation supports previous assertions (*18*) that PhE substitution for PhC enhances the interaction of the Gla-containing coagulation proteins with negatively charged lipids, and that the effect is most likely due to the bulky, hydrated quaternary ammonium ion in PhC.

The effect of PhE substitution for PhC on binding of bPt to PhS-containing monolayers demonstrates that protein affinity for membranes is not simply dependent on the density of negative charge (or PhS) in the lipid assembly. If PhE inclusion increases protein access to PhS, then a similar effect may also be operative with respect to the influence of lipid-packing density on the binding affinity. It has been firmly established, using both spread monolayers and vesicles, that increasing the lipid-packing density lowers the Ca^{2+}-specific binding affinity of Gla-containing coagulation proteins for membranes. Although this could result from a requirement for insertion of hydrophobic protein side chains into membranes (*2*), the PhE results suggest that it could also be due to limitations on access of the protein to PhS. According to the data in Figure 1, the influence of packing density is more extensive when PhC is the zwitterionic component of the lipid assembly. This is most likely related to the biocompatible nature of the PhC headgroup (*19, 20*). Thus, it is possible that the Ca^{2+}-bridging interaction involved in Gla protein−membrane contact can be modulated by the lipid-packing density.

The use of spread phospholipid monolayers and combined measurements of π and Γ have provided new and interesting information regarding protein-membrane contact. The significance of this study is that it provides the first direct experimental evidence for a similar Ca^{2+}-specific protein−membrane contact mechanism among the various vitamin K-dependent coagulation proteins. Differences in the Gla domain sequence appear not to play major roles in actual Ca^{2+}-specific protein−membrane contact, although they certainly influence the binding affinity. A recent molecular modeling study has proposed the structure of a membrane contact site in the Gla domain in the case of bilayers (*10*). Although there is some uncertainty as to the existence of more than one membrane contact site, a single-site mechanism is consistent with a common mode of attachment for the vitamin K-dependent coagulation proteins.

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