# Prothrombin Association with Phospholipid Monolayers†

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ABSTRACT: Surface pressure measurements demonstrated that prothrombin and its fragment 1 region caused minor perturbations of lipid structure upon binding to phospholipid monolayers. These perturbations did not appear to be due to insertion of the protein into the monolayer film. The actual amount of protein bound to the monolayer was measured by recovering the monolayer and the $^3$H-labeled fragment 1 associated with it. Binding was strictly dependent on calcium and acidic phospholipid. Titration of the protein–monolayer interaction indicated a single class of binding sites with a $K_D$ of 0.15 $\mu$M for the interaction of fragment 1 with 20% phosphatidylserine (PS)–80% phosphatidylcholine (PC) monolayers at an initial surface pressure of 21 dyn/cm. A small increase in binding affinity was observed at higher percentages of PS. The fragment 1 binding capacity of PS–PC monolayers was proportional to the PS content between 0 and 20% PS with a stoichiometry of 7.8 ± 0.9 PS residues per bound protein molecule. Above 20% PS, the direct proportionality was no longer observed, indicating that some theoretical binding sites were being sterically excluded. The surface concentration of fragment 1 molecules bound to 100% PS monolayers at saturating protein corresponded to one bound protein per 1077 Å$^2$. This area compared well with 1100 Å$^2$ per molecule calculated from surface pressure changes. One protein molecule, therefore, occupied an area greater than that of the required PS residues. At high surface pressures, the protein binding capacity of the monolayers remained relatively unchanged, but the surface pressure changes decreased and, in some cases, became zero. Binding affinity also decreased at higher surface pressures. Comparison of prothrombin binding to monolayers with its binding to bilayer vesicles revealed few, if any, significant differences. Thus, the radius of curvature does not appear to be an important determinant in this protein–lipid interaction, and the two systems appear complementary in the analysis of this association.

The binding of prothrombin to a membrane surface is essential for blood clotting activity [see Jackson & Nemerson (1980) for a review]. This binding involves a specific interaction between prothrombin and membranes containing acidic phospholipids via a calcium-dependent process (Papahadjopoulos & Hanahan, 1964; Bull et al., 1972). Prothrombin fragment 1, formed by the action of thrombin on prothrombin, consists of the amino-terminal, $\gamma$-carboxyglutamic acid containing region of prothrombin. Characteristics of the fragment 1–acidic phospholipid membrane association are very similar to those for intact prothrombin (Nelsestuen, 1976). Fragment 1 can therefore be viewed as a good model for prothrombin–membrane binding.

Phospholipid bilayer vesicles have been used to obtain various binding parameters for the prothrombin–membrane interaction. Fluorescence and light scattering techniques have been employed to characterize dissociation constants (Nelsestuen & Broderius, 1977; Bloom et al., 1979), orientation and mode of binding (Nelsestuen & Lim, 1977), acidic phospholipid–protein stoichiometry (Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977), and phospholipid reorganization upon protein–membrane binding (Mayer & Nelsestuen, 1981). The binding of prothrombin fragment 1 to vesicles has also been studied by using quite different techniques (Dombrose et al., 1979), yielding very similar results. Other investigators have characterized the binding of prothrombin to collapsed phospholipid surface films at the air–water interface. Adsorption to these films, which contained 3 times the lipid necessary to form a lipid monolayer, was monitored by using surface radioactivity and electrical capacitance techniques (LeCompte et al., 1980; LeCompte & Miller, 1980). The reported differences in the adsorption of prothrombin to these films as compared to bilayers suggested that curvature of the interface might be an important determinant of this lipid–protein association. To better define these possible differences and the adsorption process itself, we have studied the adsorption of prothrombin and its fragment 1 to monomolecular lipid films. Adsorption of prothrombin to monolayers caused a small but measurable change in surface pressure. As a result, the system displayed near-ideal behavior and was highly amenable to these types of studies. Under these well-defined conditions, the adsorption parameters obtained compared favorably with those obtained with bilayers, indicating the absence of curvature effects. Furthermore, adsorption appears to occur almost exclusively through interactions with the phosphatidylserine (PS)[1] head groups with minimal perturbation of the hydrocarbon region of the lipid molecules.

## Materials and Methods

Bovine prothrombin was initially purified by a modification (Nelsestuen & Suttie, 1973) of the method of Ingwall & Scheraga (1969). Final purification was accomplished by passing a concentrated prothrombin solution through a Bio-Gel A 0.5M column (3 × 100 cm). Peak fractions were pooled and stored at −80 °C until use. Prothrombin fragment 1 was produced by the action of thrombin on prothrombin, followed by isolation as described by Heldebrant & Mann (1973). This preparation was then concentrated and passed through a Sephadex G-100 superfine column (4.5 × 150 cm). Again, peak fractions were pooled and stored at −80 °C without

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[1] Abbreviations: PS, bovine brain phosphatidylserine; PC, egg yolk phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; $\pi$, $\Delta\pi$, and $\pi_0$, actual surface pressure, surface pressure change, and initial surface pressure, respectively; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/83/0422-0316$01.50/0 

further manipulation. $^3$H-Labeled fragment 1 was prepared by the method of Van Lenten & Ashwell (1971) followed by ion exchange and gel filtration chromatography described for native fragment 1. A specific activity of 1800 cpm/$\mu$g of protein was obtained. Proteins were quantitated by the 280-nm absorbance as previously described (Resnick & Nelsestuen, 1980).

Bovine brain phosphatidylserine and egg yolk phosphatidylcholine were purchased from Sigma Chemical Co. Further purification of these lipids by high-pressure liquid chromatography (Varian LC 5000) was accomplished by using a Varian CN-10 column and a low-water variation of the solvent gradient system described by Hax & Geurts Van Kessel (1977). The elution solvent was dried under $N_2$, and the purified lipids were resuspended in redistilled hexane (PC) or chloroform (PS), flushed with argon, and stored at −80 °C until use. Phospholipid concentrations were determined by organic phosphate measurement (Chen et al., 1956) with a phospholipid/phosphorus weight ratio of 25. Careful attention to the purity of the protein and phospholipid was necessary in order to obtain consistent results.

Monolayers were formed by applying the appropriate lipids dissolved in ethanol–hexane (10:90) onto buffer contained in a 10-mL (4 × 0.8 cm) circular Teflon trough etched with Teflon-treating agent (Chemplast Inc., Wayne, NJ). A small port in the side of the trough enabled injection of reagents into the subphase without disruption of the monolayer. Unless indicated, the buffer consisted of 0.05 M Tris, 0.1 M NaCl, and 10 mM $CaCl_2$ (pH 7.5). The subphase was gently mixed with a Teflon stir bar at 45 rpm. Surface pressure measurements were obtained by using the Whilhelmy plate method employing a mica plate. The plate was cleaned with concentrated chromic–sulfuric acid and thoroughly rinsed with double-distilled water prior to each surface pressure measurement. Surface pressure–molecular area isotherms for mixtures of PS and PC were determined at 24 °C by using a Langmuir-type film balance (Brockman et al., 1980). The compression rate was 5–8 Å$^2$ min$^{-1}$ molecule$^{-1}$.

Amounts of $^3$H-labeled fragment 1 bound to phospholipid monolayers were determined as follows: the phospholipid film was spread and allowed to reach equilibrium (1 h). Appropriate reagents were then injected into the subphase and allowed to equilibrate. Whatman 1 PS hydrophobic paper was used to recover the monolayer as previously described (Bhat & Brockman, 1981). Ninety percent of the monolayer is recovered by this method. The paper was cut into small segments and placed into a scintillation vial along with 1 mL of 0.02 M EDTA (which releases protein from the paper) and 10 mL of scintillation fluid. Backgrounds were determined by recovering pure PC monolayers in the presence of various amounts of $^3$H-labeled fragment 1. The background radioactivity recovered was nonsaturable and was directly proportional to the amount of protein in the subphase. This background corresponded to 48 ± 6 $\mu$L of subphase obtained with each recovered monolayer and ranged from 10 to 70% of the total counts isolated in the experiments given here. Values reported below have been corrected for percent recovery and subphase background. Direct determination of subphase radioactivity indicated no significant protein loss to the Teflon material.

## Results

*Monolayer Stability and Miscibility of PS and PC.* As measured by the Whilhemly technique, the equilibrium spreading pressures of the pure lipids were 46.5 dyn/cm for PC and 41.5 dyn/cm for PS. The collapse pressures of the

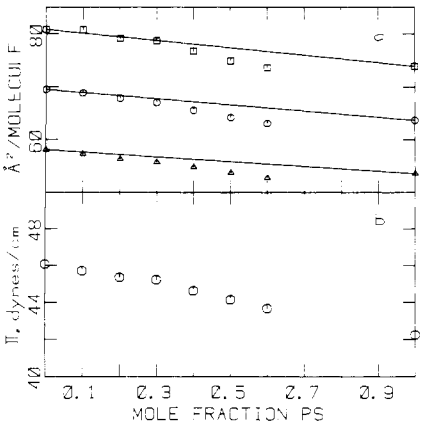


FIGURE 1: (a) Average molecular area as a function of composition for phosphatidylserine–phosphatidylcholine mixtures. Pressures represented are (□) 10, (○) 20, and (△) 40 dyn/cm. (b) Composition dependence of phase transition pressures for phosphatidylserine–phosphatidylcholine mixtures. Data for both panels were obtained from surface pressure–area isotherms.

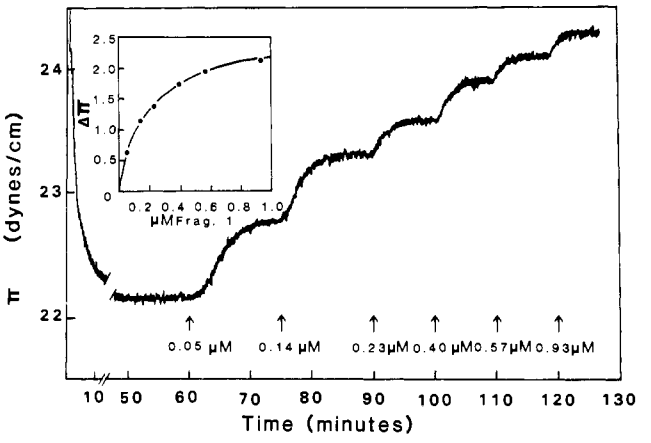


FIGURE 2: Surface pressure changes related to binding of fragment 1 to PS–PC monolayers. Surface pressure changes between 0 and 60 min are due to solvent evaporation and subsequent monolayer stabilization. The arrows indicate injection of protein to achieve the given subphase concentration. The inset is a plot of the surface pressure increase vs. fragment 1 concentration.

films obtained from surface pressure–area isotherms varied continuously between these limits as a function of composition (Figure 1b). This indicates two-dimensional miscibility of the lipids (Crisp, 1949). Miscibility is also indicated by the nonadherence of average molecular composition isobars to the additivity rule (Gaines, 1966). This behavior is exemplified in Figure 1a, which shows isobars for 10, 20.0, and 40.0 dyn/cm. Thus, at all compositions and surface pressures up to at least 41 dyn/cm, stable PC–PS films of known interfacial composition can be formed for measurements of protein adsorption.

*Surface Pressure Changes Induced by Prothrombin and Fragment 1 Binding to PS–PC Monolayers.* Injection of fragment 1 under a PS-containing monolayer caused an increase in the surface pressure of the lipid film (Figure 2). This increase was strictly dependent on calcium; neither fragment 1 nor prothrombin caused an observable surface pressure increase for PS–PC (20:80) or pure PS monolayer when injected into a calcium-free subphase. A total surface pressure increase of 2.1 dyn/cm above the initial value of 22.2 dyn/cm was observed at 0.93 $\mu$M fragment 1 in the presence of 10 mM $CaCl_2$. Under similar conditions, 0.5 $\mu$M prothrombin induced a 1.6 dyn/cm increase above an initial pressure of 23.5 dyn/cm (data not shown).

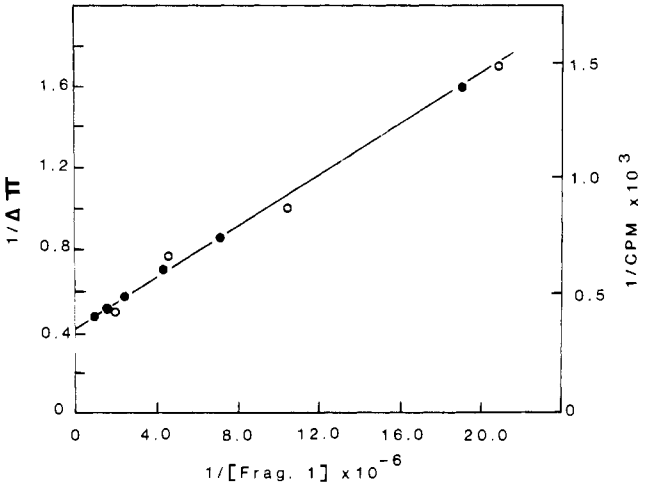


FIGURE 3: Analysis of binding data by the Langmuir relation (●). Double-reciprocal plot for $^3$H-labeled fragment 1 binding to monolayers (○). Each point represents bound protein estimated as described under Materials and Methods. In both cases, 20/80 PS/PC was used at an initial spread pressure of 21 ± 1 dyn/cm.

Table I: Dissociation Constants for Prothrombin and Fragment 1 Binding to PS-Containing Monolayers

| protein | % PS | $\pi_0$ (dyn/cm) | $K_D$ ($\mu$M) |
|---|---|---|---|
| fragment 1 | 10 | 19.15 | 0.25[a] |
| fragment 1 | 20 | 21.30 | 0.15[a] |
| $^3$H-labeled fragment 1 | 20 | 21 ± 1 | 0.16[b] |
| $^3$H-labeled fragment 1 | 20 | 41 ± 2 | 0.57[b] |
| fragment 1 | 40 | 19.65 | 0.12[a] |
| fragment 1 | 80 | 23.50 | 0.11[a] |
| prothrombin | 20 | 23.50 | 0.07[b] |

[a] Dissociation constants were obtained by titrating surface pressure change with protein addition as described under Materials and Methods. [b] Dissociation constants were obtained by titrating amount of bound radioactivity with protein addition as described under Materials and Methods.

Titration of the surface pressure increase was accomplished by adding successive amounts of fragment 1 to the subphase while continuously monitoring the surface pressure of the film (Figure 2). The observed pressure changes displayed saturation behavior as shown in the inset of Figure 2. For such small changes in surface pressure, it can be assumed that the surface structure is not significantly altered and, therefore, that the surface concentration of protein, $\Gamma$, is proportional to the change in surface pressure, $\Delta\pi$. If so and if adsorption is saturable according to the Langmuir adsorption isotherm, then (Pethica, 1955)

$$\frac{1}{\Delta\pi} = \frac{1}{\Delta\pi_m}(1 + K_d/C) \quad (1)$$

where the subscript m refers to the value of $\Delta\pi$ at saturation with protein, $C$ is the free protein concentration, and $K_d$ is a dissociation constant. In all cases, adsorbed protein did not significantly deplete the subphase. Free protein concentration was therefore equal to its total concentration. The results were consistent with eq 1, yielding $K_d$'s of 0.15 $\mu$M for the data shown (Figure 3) and 0.07 $\mu$M for prothrombin under comparable concentrations (Table I). The maximal surface pressure changes were 2.30 and 1.85 dyn/cm, respectively. Chelation of calcium with EDTA caused complete reversal of protein-induced pressure changes.

Corresponding titrations were conducted with $^3$H-labeled fragment 1 by using 20% PS–80% PC monolayers at approximately 20 dyn/cm. Radiolabeled fragment 1 was indistiguishable from native fragment 1 with respect to its

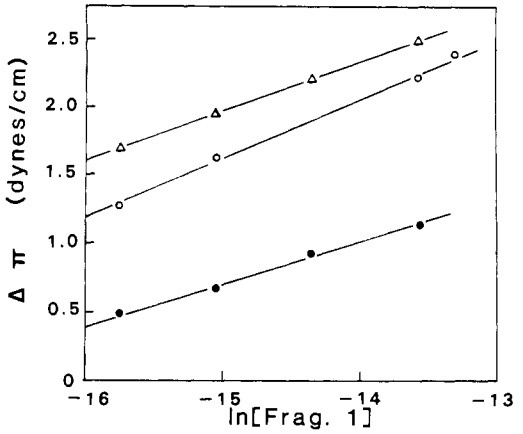


FIGURE 4: Gibbs plot for fragment 1 binding to PS–PC monolayers ($\pi_0$ = 21 ± 1 dyn/cm): (●) 10/90 PS/PC; (○) 20/80 PS/PC; (△) 40/60 PS/PC.

dissociation constants and maximum surface pressure change (Table I). The actual amount of $^3$H-labeled fragment 1 bound to the monolayer was also determined as described under Materials and Methods. A double-reciprocal plot of bound and free $^3$H-labeled fragment 1 (Figure 3) gave a dissociation constant of 0.16 $\mu$M when 20% PS–80% PC monolayers were used at an initial surface pressure of 20 dyn/cm. Thus, at constant film pressures, the amount of surface pressure increase was proportional to the actual amount of fragment 1 bound to the monolayer (Figure 3) as assumed above.

Dissociation constants for fragment 1 binding to 10, 40, and 80% PS monolayers were also obtained by monitoring surface pressure changes. The results in Table I illustrate a small dependence of the dissociation constant on the mole percent PS in the monolayer. The relationship between percent PS and the dissociation constant is very similar to prothrombin's association with bilayer vesicles at saturating calcium concentration (Nelsestuen & Broderius, 1977; Figure 2).

*Binding Isotherms of Fragment 1 and Prothrombin.* Data from surface pressure titrations (Figure 1) of 10, 20, and 40% PS monolayers were analyzed by the modified Gibbs equation (Pethica, 1955):

$$d\pi = \phi\Gamma RT\, d \ln C \quad (2)$$

where $\Gamma$ is the maximal surface excess of protein if the lipid does not compete with protein for space at the interface. Figure 4 shows that such plots are linear for fragment 1 adsorption to monolayers containing 10, 20, and 40 mol % PS. From each slope, $\phi\Gamma$ can be calculated, and its reciprocal is the molecular area of the protein at saturation. The values obtained were 1300, 975, and 1050 Å$^2$/molecule, respectively. Thus, the area of the binding site appeared unchanged with PS–PC composition, and an average area of 1100 Å$^2$ per fragment 1 binding site was obtained. The same plot for prothrombin binding to 20% PS–80% PC monolayers was also linear and yielded an area of 1250 Å$^2$ per binding site. Surface pressure–area isotherms determined for PC, PS, and mixed PS–PC monolayers indicated that an initial pressure of 21 dyn/cm gave an average area per phospholipid molecule of 67 Å$^2$. A binding site area of 1100 Å$^2$ would therefore indicate that approximately 16 phospholipid molecules were "covered" when one fragment 1 molecule bound to the monolayer surface.

*Fragment 1 Binding to Monolayers as a Function of PS–PC Composition.* Surface pressure increases and amounts of fragment 1 bound at nearly saturating concentrations were determined as a function of mole percent PS (Figure 5). Each point represents a single determination at a fragment 1 con-

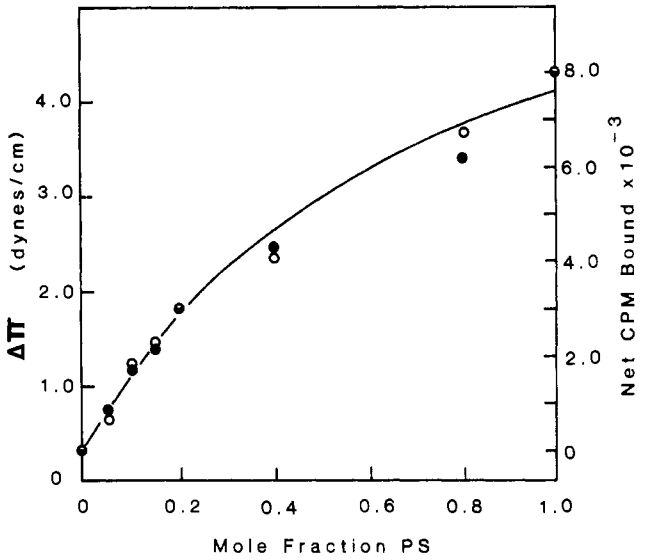


FIGURE 5: Dependence of surface pressure increase and amount of bound protein on PS–PC composition. Monolayers were spread at an initial pressure of 21 ± 1 dyn/cm. Fragment 1 (closed symbols) and $^3$H-labeled fragment 1 (open symbols) were injected to achieve a concentration 10 times the $K_D$ for the monolayer composition used. Pressure change (●) and bound radioactivity (○) were measured.

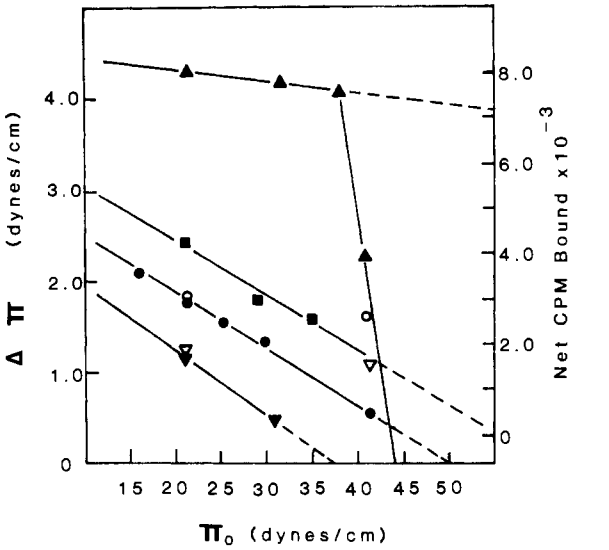


FIGURE 6: Dependence of surface pressure increase and amount of bound protein on the initial spread pressure of PS–PC monolayers. Fragment 1 or $^3$H-labeled fragment 1 was injected to achieve a concentration 10 times the $K_D$ for the monolayer composition used. Pressure changes for (▼) 10/90 PS/PC, (●) 20/80 PS/PC, (■) 40/60 PS/PC, and (▲) pure PS monolayers are shown. Bound radioactivities for (▽) 10/90 PS/PC and (○) 20/80 PS/PC are also shown.

centration approximately 10 times the $K_D$ for that PS–PC mixture. An initial film pressure of 21 ± 1.0 dyn/cm was used for all experiments in Figure 5. Identical amounts of phospholipid were spread for cases where the amount of $^3$H-labeled fragment 1 bound to the monolayer was to be determined. The results demonstrate once again that the surface pressure increase was proportional to the actual amount of fragment 1 bound to monolayers at constant and low initial surface pressures. Pure PC monolayers (Figure 5) provided the true control for this system. The absence of bound protein and surface pressure changes indicated that surface denaturation did not occur under the conditions used here. Increasing the percent PS in the monolayer resulted in an increase in the surface pressure change as well as the amount of bound $^3$H-labeled fragment 1. This increase was approximately linear between 0 and 20% PS. The ratio of PS molecules to protein binding sites was calculated from this linear region and gave a stoichiometry of 7.8 ± 0.9 PS molecules per fragment 1 binding site.

Above 20% PS, the amount of fragment 1 bound was no longer proportional to the number of PS molecules present, indicating that some theoretical binding sites were sterically excluded. An area of 1077 Å$^2$ per fragment 1 molecule bound was obtained by using the saturating amount of $^3$H-labeled fragment 1 bound to pure PS monolayers at an initial surface pressure of approximately 20 dyn/cm (Figure 5) and the total surface area of the 4-cm diameter trough. This value compares favorably with the area per binding site obtained from the modified Gibbs equation (1100 Å$^2$, Figure 4). The binding of fragment 1 to pure PS (Figure 5) therefore appears to represent sterically saturated protein binding.

*Effects of Initial Surface Pressure on $\Delta\pi$ and on the Binding of Fragment 1 to PS-Containing Monolayers.* Surface pressure increases induced by fragment 1 binding and the actual amounts of bound fragment 1 were determined for monolayers at various initial surface pressures (Figure 6). Again, each point represents one addition of fragment 1 to the subphase to give a concentration approximately 10 times the $K_D$ for protein binding to the PS–PC mixture used. Extrapolation of this curve to $\Delta\pi_0 = 0$ gives the critical pressure for monolayer perturbation by protein binding (Bougis et al., 1981). Above the critical pressure, protein binding caused no increase in the surface pressure. The critical pressure varied extensively with the percent PS in the monolayer. Above 10% PS, the critical pressure was higher than the collapse pressure (the pressure at which the monolayer is at maximum packing density) of about 44 dyn/cm. The results for pure PS monolayers demonstrated that while the critical pressure may be much higher than the collapse pressure, no actual surface pressure change was observed above 44 dyn/cm.

The actual adsorption data obtained with $^3$H-labeled fragment 1 indicated that surface pressure changes were not proportional to bound protein when the initial surface pressure was varied. Fragment 1 need not induce any pressure change when it binds to the monolayer (see bound radioactivity at 10% PS, $\pi_0$ = 41 dyn/cm). Only small changes in the total amount of fragment 1 bound to 10 or 20% PS monolayers occurred between 21 and 41 dyn/cm. In the same range, however, the surface pressure increase was abolished for 10% PS and was reduced dramatically for 20% PS monolayers. Also, the dissociation constant for fragment 1 binding to 20% PS–80% PC monolayers increased 3.5-fold between 21 and 41 dyn/cm (Table I).

## Discussion

The small surface pressure increases induced by fragment 1 binding to monolayers at an initial surface pressure of approximately 20 dyn/cm are indicative of minor perturbation of the monolayer structure rather than of protein insertion into the lipid film. The latter commonly gives rise to much greater surface pressure increases (Bougis et al., 1981). The inability of fragment 1 to induce a significant change in the surface pressure of pure PC monolayers at an initial pressure of 21 dyn/cm (Figure 5) and its ability to bind without causing a surface pressure increase (Figure 6, 10% PS) also argue against protein insertion. The pressure increases were dependent on calcium; injection of prothrombin or fragment 1 under 20/80 PS/PC or pure PS monolayers in the absence of calcium did not alter the surface pressure of the film. The interaction of prothrombin and fragment 1 with PS-containing monolayers appears to involve calcium-dependent binding to the head

group of acidic phospholipids. The surface pressure increases observed upon protein binding may be due to a protein-induced change in the phospholipid packing.

Prothrombin and fragment 1 adsorption to monolayers obeyed the Langmuir adsorption isotherm (eq 1) with a single binding constant. Dissociation constants for prothrombin and fragment 1 binding to 20/80 PS/PC monolayers at 20 dyn/cm were 0.07 and 0.15 $\mu$M, respectively. This small difference between prothrombin and fragment 1 was observed previously with bilayer vesicles (Nelsestuen & Lim, 1977). The magnitude of the difference is small and has not been investigated further. Increasing the percent PS in the monolayer caused a small decrease in the dissociation constant. These properties were also consistent with those obtained with bilayer phospholipid vesicles at saturating calcium concentrations (Nelsestuen & Broderius, 1977; Resnick & Nelsestuen, 1980) except that binding affinities were somewhat tighter for monolayers spread at 21 dyn/cm than for vesicles. This difference is apparently due to the initial surface pressure; the dissociation constant obtained at 41.6 dyn/cm for 20/80 PS/PC monolayers (0.57 $\mu$M, Table I) is very comparable to the data for bilayer vesicle systems ($K_D$ = 0.3–0.4 $\mu$M; Resnick & Nelsestuen, 1980). The phospholipid packing density of bilayer vesicles closely resembles that for monolayers at the collapse pressure. Thus, the actual binding affinity of fragment 1 for 20/80 PS/PC monolayers appears very similar to its binding to bilayer vesicles of the same composition.

Fragment 1 binding to PS-containing monolayers occurred close to the collapse pressure even though surface pressure changes were small (Figure 6, 20–100% PS) or absent (Figure 6, 10% PS). At high surface pressures (above 40 dyn/cm) and high PS content, the data suggested that the phospholipid film may have collapsed upon protein binding. For example, although protein binding to 100% PS at 41 dyn/cm appeared capable of inducing a 4 dyn/cm change (Figure 6), this would exceed the collapse pressure of the monolayer and was probably compensated by loss of phospholipid from the film. At high surface pressures and low PS content (Figure 6, 10% PS), the binding of fragment 1 did not cause an increase in surface pressure, and the monolayer packing density was apparently unchanged. While the binding capacity of PS-containing monolayers remained relatively unchanged (Figure 6), binding affinity decreased (Table I) as the initial surface pressure was increased from 20 to 40 dyn/cm.

The average area per fragment 1 binding site was 1100 Å$^2$ (calculated by using the Gibbs equation and the data from Figure 5). The binding area for prothrombin has been estimated previously by using phospholipid vesicles (Lim et al., 1977; Dombrose et al., 1979). While the areas per binding site were somewhat larger (1800 Å$^2$; Lim et al., 1977), the results with monolayers reported here appear to provide a better estimate of this parameter. Pure PS monolayers could be used to obtain a fully saturated membrane, and the Gibbs equation provided an independent estimation.

The stoichiometry of 7.8 ± 0.9 PS residues per bound fragment 1 molecule is in agreement with values obtained for vesicle systems (9 ± 1 PS; Nelsestuen & Broderius, 1977). At an initial surface pressure of 20 dyn/cm, approximately 16 phospholipid residues were covered by a single fragment 1 molecule. Consequently, the protein covers an area greater than that of the acidic phospholipids involved in the binding. Two models can be considered for the structure of the binding site. The first consists of a cluster of eight acidic phospholipids surrounded by noninteracting phospholipids. This model is supported by previous studies indicating that acidic phospholipid clustering occurs upon prothrombin binding to PS–PC vesicles (Mayer & Nelsestuen, 1981). The second model consists of a binding site with eight acidic phospholipids interspersed with noninteracting phospholipids. Below a PS/PC ratio of 1/1, both models would entail some clustering of acidic phospholipids within the binding domain.

The interaction of prothrombin and fragment 1 with phospholipid multilayers was investigated previously by using surface radioactivity and polarographic techniques (LeCompte & Miller, 1979, 1980; LeCompte et al., 1980). About 3 times the lipid needed to achieve the collapse pressure was applied. Therefore, data obtained here at high initial surface pressures should be used for comparison with the earlier results. The dissociation constant of 0.57 $\mu$M for fragment 1 binding to 20% PS–80% PC monolayers ($\pi_0$ = 41 dyn/cm, Table I) compares favorably to the value of about 0.32 $\mu$M determined for fragment 1 binding to monolayers of similar composition and at high calcium concentrations (LeCompte et al., 1980). Qualitatively, tighter binding at higher PS content was observed in both studies. However, the reported affinity of fragment 1 for pure PS monolayers (LeCompte et al., 1980) was about 50 times greater than that obtained in this study. The amount of bound protein was also about 2 times greater in the previous study. The reason for these discrepancies is not known, although several differences in methodology exist. The previous studies used excess lipid to achieve a condensed film. Surface pressures were not obtained, and the actual amount of phospholipid at the air–water interface was not determined. Ohki & Duzgunes (1979) showed that pure phosphatidylserine in the subphase can interact with a monolayer in the presence of calcium. Such interactions could give higher levels of surface radioactivity determined by external measurement techniques (LeCompte et al., 1980). Our studies utilized monolayers containing known amounts of phospholipid with little or no lipid in the subphase. Surface pressure and radioactivity actually bound to the monolayer were measured and gave corroborating results. LeCompte et al. (1980) also reported prothrombin binding to monolayers in the absence of calcium. We did not observe this by using highly purified protein at the concentrations reported here.

Characteristics of protein association with PS-containing phospholipid monolayers or bilayer vesicle systems appear very similar. Surface curvature apparently does not significantly alter the binding characteristics. The two systems appear strictly complementary for analyzing binding parameters important in this protein–membrane association. The present monolayer study provided several unique observations in addition to corroboration of several findings made with bilayer vesicle systems.

Acknowledgments

We thank William Momsen for his excellent technical instruction.

**Registry No.** Prothrombin, 9001-26-7; calcium, 7440-70-2.

References

Bhat, S. G., & Brockman, H. L. (1981) *J. Biol. Chem. 256*, 3017.

Bloom, J. W., Nesheim, M. E., & Mann, K. G. (1979) *Biochemistry 18*, 4419.

Bougis, P., Rochat, H., Pieroni, G., & Verger, R. (1981) *Biochemistry 20*, 4915.

Brockman, H. L., Jones, C. M., Schwebke, C. J., Smaby, J. M., & Jarvis, D. E. (1980) *J. Colloid Interface Sci. 78*, 502.

Bull, R. K., Jevons, S., & Barton, P. G. (1972) *J. Biol. Chem. 247*, 2747.

Crisp, D. J. (1949) *Surface Chemistry*, pp 17-22, Butterworths, London.
Dombrose, F. A., Gitel, S. N., Zawalich, K., & Jackson, C. M. (1979) *J. Biol. Chem. 254*, 5027.
Gaines, G. L., Jr. (1966) *Insoluble Monolayers at Gas-Liquid Interfaces*, pp 281-286, Wiley, New York.
Hax, W. M. A., & Geurts Van Kessel, W. S. M. (1977) *J. Chromatogr. 142*, 735.
Heldebrant, C. M., & Mann, K. G. (1973) *J. Biol. Chem. 248*, 3642.
Ingwall, J. S., & Scheraga, H. A. (1969) *Biochemistry 8*, 1860.
Jackson, C. M., & Nemerson, Y. (1980) *Annu. Rev. Biochem. 46*, 767.
LeCompte, M. F., & Miller, I. R. (1979) *Bioelectrochem. Bioenerg. 6*, 537.
LeCompte, M. F., & Miller, I. R. (1980) *Biochemistry 19*, 3439.
LeCompte, M. F., Miller, I. R., Elion, J., & Benarous, R. (1980) *Biochemistry 19*, 3434.
Lim, T. K., Bloomfield, V. A., & Nelsestuen, G. L. (1977) *Biochemistry 16*, 4177.
Mayer, L. D., & Nelsestuen, G. L. (1981) *Biochemistry 20*, 2457.
Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648.
Nelsestuen, G. L., & Suttie, J. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3366.
Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16*, 4172.
Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164.
Ohki, S., & Duzgunes, N. (1979) *Biochim. Biophys. Acta 552*, 438.
Papahadjopoulos, D., & Hanahan, D. J. (1964) *Biochim. Biophys. Acta 90*, 438.
Pethica, B. A. (1955) *Trans. Faraday Soc. 51*, 1402.
Resnick, R. M., & Nelsestuen, G. L. (1980) *Biochemistry 19*, 3028.
Van Lenten, L., & Ashwell, G. (1971) *J. Biol. Chem. 246*, 1889.

# Measurement of the Refolding Combination Reaction between S-Peptide and S-Protein†

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ABSTRACT: S-Peptide combines with S-protein during the refolding of ribonuclease S. The kinetics of combination have now been measured by a specific probe, the absorbance (492 nm) of a fluoresceinthiocarbamyl (FTC) group on lysine-7 of S-peptide. p$K$ changes of the FTC group detect both initial combination and later, first-order, stages in folding. Combination with the slow-folding species of S-protein occurs with a half-time of 0.4 s at 50 $\mu$M, whereas complete folding takes 50 s (pH 6.8, 31 °C). Thus combination takes place at an early stage in folding. The second-order rate constant of the refolding combination reaction ($5 \times 10^4$ $M^{-1}$ $s^{-1}$) is 100-fold smaller than that for combination with folded S-protein, which probably reflects the lower affinity of S-protein for S-peptide in the initial complex. Inhibition by S-peptide of combination between FTC-S-peptide and S-protein shows that the refolding combination reaction is specific and reversible. Both the fast-folding and slow-folding species of unfolded S-protein participate in the refolding combination reaction.

Ribonuclease S (RNase S)[1] dissociates into S-protein plus S-peptide upon unfolding, and these reassociate during refolding (Richards & Logue, 1962; Labhardt & Baldwin, 1979a). This property can, in principle, be used to characterize the site at which combination occurs during folding and to determine the interactions responsible for combination. This may be an effective way of studying how recognition takes place between different parts of a protein during folding. The first step in this work is to find a probe that detects combination during folding and then to measure the kinetics of combination. Initial combination is not detected by changes in tyrosine absorbance or fluorescence in the refolding of unlabeled RNase S (Labhardt & Baldwin, 1979a), and the refolding combination reaction has also not yet been observed by circular dichroism (A. M. Labhardt, unpublished results). We report here that an FTC group covalently attached to the $\epsilon$-$NH_2$ group of Lys-7 by the Edman reaction provides a probe that detects the refolding combination reaction.

Unfolded RNase S contains both fast-folding ($U_F$) and slow-folding ($U_S$) species, as does RNase A. The $U_F$ and $U_S$ species have different refolding pathways that have been

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; RNase S, a derivative of RNase A in which the peptide bond between residues 20 and 21 has been cleaved; S-peptide, residues 1-20 of RNase S; S-protein, residues 21-124 of RNase S; FTC, fluoresceinthiocarbamyl; FTC-S-peptide, des-1,7$^t$-FTC-S-peptide; FTC-RNase S, FTC-S-peptide combined with S-protein; $U_F$ and $U_S$, fast-folding and slow-folding forms of an unfolded protein; p, S-peptide (or FTC-S-peptide); pN, native RNase S (or FTC-RNase S); $A$, absorbance; GdmCl, guanidinium chloride; $t_m$, temperature midpoint of a thermal unfolding transition; $\tau$, time constant of a reaction (reciprocal of the apparent rate constant); C>p, cytidine 2',3'-phosphate; 2'-CMP, cytidine 2'-phosphate; $k_{cat}$, turnover number; $K_m$, Michaelis-Menten constant; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; CM, carboxymethyl.

0006-2960/83/0422-0321$01.50/0 