Henriksen, R. A., & Jackson, C. M. (1975) *Arch. Biochem. Biophys. 170*, 149-159.
Jackson, C. M., Peng, C. W., Brenckle, G. M., Jonas, A., & Stenflo, J. (1979) *J. Biol. Chem. 254*, 5020-5026.
Lecompte, M. F., & Miller, I. R. (1980) *Adv. Chem. Ser.* (in press).
Mann, K. G. (1976) *Methods Enzymol. 45*, 123-156.
McLaughlin, S. G. A., Saubo, G., & Eisenman, G. (1971) *J. Gen. Physiol. 58*, 667-687.
Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648-5656.
Nelsestuen, G. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 2621-2625.
Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16*, 4172-4176.
Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164-4171.
Nelsestuen, G. L., Broderius, M., & Martin, G. (1976) *J. Biol. Chem. 251*, 6886-6893.
Ohki, S., & Sauve, R. (1978) *Biochim. Biophys. Acta 511*, 377-387.
Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840-850.

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

M. F. Lecompte‡ and I. R. Miller*

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

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

† From the Department of Membrane Research, Weizmann Institute of Science, Rehovot, Israel. *Received October 22, 1979.* The work of M.F.L. was supported by INSERM Grant No. 805009 through the host institute, Laboratoire d'Electrochimie Interfaciale, Laboratoire de Bellevue CNRS.

‡ On leave of absence from the Laboratoire de Recherches sur les Macromolécules, directed by Dr. M. Jozefowicz, C.S.P., Université Paris-Nord, 93430 Villetaneuse, France.

0006-2960/80/0419-3439$01.00/0

$$RSSR + Hg \rightleftarrows (RS)_2Hg \text{ or } (RS)_2Hg_2 \xrightarrow{e + H^+} RSH + Hg \quad (1)$$

The cysteine formed during reduction tends to desorb, and the redox reaction may be accompanied by adsorption or desorption of the cystine and cysteine residues, respectively. This adsorption–desorption process involves only parts of the whole protein molecule attached to the monolayer. The rate of evolvement of the pseudocapacitance peak after setting the anodic potential is relatively slow (minutes), and it involves gross structural changes of the protein within the lipid layer. The ac frequency dependence of the pseudocapacitance peak reflects relatively fast (millisecond to second region) dynamic structural fluctuations involving adsorption–desorption of a few residues of the surface complex only.

In an attempt to elucidate what parts of the prothrombin molecule contribute to the hydrophobic interaction and penetration, we decided to investigate the effect of its three molecular fractions on the capacitance of the phospholipid monolayers. Any conclusions reached from the experiments are to be taken with caution since the conformation of the isolated fractions is not necessarily identical with their tertiary structure in the prothrombin molecule, even though they preserve their secondary structure (Bloom & Mann, 1979). Moreover, fragment II and thrombin are devoid of γ-carboxyglutamic acid residues to enhance their adsorption. Since penetration is proportional to the local concentration in the surface, the contribution of these fragments to penetration of prothrombin when attached to the surface with the aid of the γ-carboxyglutamic acid residues in fragment I may be larger than inferred from their effect in the isolated state, on the lipid layer capacitance. The interaction of the segments with the lipid layer is, however, important per se, since these fragments are displaced from the surface upon completion of the enzymatic cleavage.

Experimental Section

Fragment I and fragment II of prothrombin and thrombin were purified by J. Elion (Institut de Pathologie, 75014 Paris, France).

All the other materials used are described in the preceding paper (Lecompte et al., 1980). Ac polarography and cyclic voltametry measurements were carried out on a P.A.R. Model 170 polarographic instrument. The electrodic investigation was carried out in a Metrohm polarographic cell filled with buffered (e.g., 10^{-3} M Tris, pH 7.8) 0.1 M NaCl solution. The surface area of the solution in the cell was about 30 cm^2. After air oxygen was displaced by nitrogen, the compressed lipid monolayer was formed by spreading a two- to threefold excess of lipid. The excess lipid formed collapsed structures in equilibrium with the spread monolayer. It had a negligible contribution to protein adsorption and none at all to the electrodic measurements. The prothrombin and Ca^{2+} were injected underneath the lipid monolayer to the desired concentrations. Equilibration between the bulk and the surface layer was facilitated by gentle stirring of the subphase with a magnetic stirrer.

At a time t when equilibrium has been reached in the adsorption determination by surface radioactivity, a hanging mercury drop was formed on a capillary tip of an electrode device positioned about 0.2 mm above the monolayer. Thus, the monolayer transferred on the mercury electrode–water interface stayed in equilibrium with the monolayer reservoir on the air–water interface. Measurements were performed after different exposure times (15 s to 20 min) of the monolayer to the electrode at the given potential.

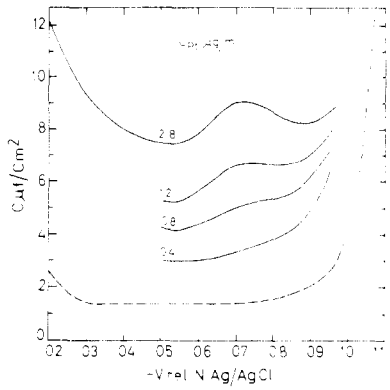

FIGURE 1: Differential capacity of condensed phosphatidylserine monolayer, with different concentrations of prothrombin injected underneath at 0.5 mM Ca^{2+}, as a function of the mercury electrode potential. The dotted line represents the phosphatidylserine monolayer in the presence of Ca^{2+}.

The dropping mercury electrode contacting the monolayer, as described in previous publications (Miller et al., 1976; Miller & Rishpon, 1977), gives erratic results when the monolayer becomes rigid upon interaction with prothrombin in the presence of Ca^{2+} and does not adjust readily to the expanding mercury surface. A silver|silver chloride electrode in 1 N KCl was the reference electrode, and a platinum gauge was the auxiliary electrode in our three electrodes system. Cyclic voltametry was performed at different sweep rates, between 0.02 and 2 V/s. In ac polarography, the potential was scanned at a rate of 20 mV/s, and the frequency of the ac modulation (10 mV peak to peak) was varied between 10 and 1000 Hz. The starting potentials were chosen to be in the stable region of the monolayer, namely between −200 and −900 mV relative to the 1 N Ag|AgCl electrode.

Results

(1) Effect of Proteins on Capacitance of Lipid Monolayers. *(a) Prothrombin.* The effect was measured by ac polarography. The effect of different concentrations of prothrombin in the presence of 0.5 mM Ca^{2+} on the differential capacitance as measured by ac polarography at 80 Hz is given in Figure 1. The overall capacitance as well as the size of the pseudocapacitance peak around 0.7 V increase with the concentration of prothrombin until about 3 μg/mL. Above this concentration there is almost no further change in capacitance. The starting potential in the experiments presented in Figure 1 was −0.2 or −0.5 V relative to the 1 N Ag|AgCl electrode. The monolayer was exposed to this potential for up to 10 min. At 0.5 V the monolayer is most stable, and the potential is remote enough from the cystine–cysteine redox pseudocapacitance peak potential. The capacitance variation at this potential was almost independent of the starting potential and was, therefore, selected to represent the effect of the protein penetration into the lipid layer on capacitance. The increase in capacitance with prothrombin concentration in the presence of different concentrations of Ca^{2+} is presented in Figure 2. Even in the absence of Ca^{2+} there is an increase in capacitance upon addition of prothrombin. However, the increase with concentration is less steep in the low Ca^{2+} concentration region. In every case the initial increase in capacitance is proportional to the increase in surface concentration in this region as determined by surface radioactivity (Lecompte & Miller, 1980). This proportionality continues up to a surface concentration of about 1.5×10^{-12} mol/cm^2. This surface concentration is reached at lower bulk concentration in the high Ca^{2+} than in the low Ca^{2+} region. Above this surface concentration, the

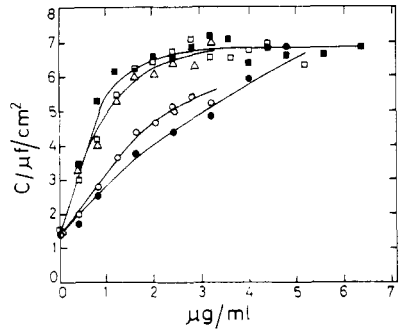

FIGURE 2: Differential capacity of condensed phosphatidylserine monolayer, at −0.5 V relative to 1 N Ag|AgCl electrode, as a function of the prothrombin concentration, at different Ca^{2+} concentrations: (●) 0; (○) 10^{-3}; (△) 0.1 mM; (□) 0.5 mM; (■) 2 mM.

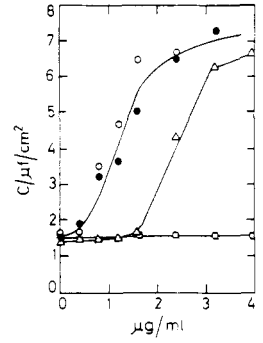

FIGURE 3: Differential capacity of condensed monolayer containing 75% phosphatidylcholine + 25% phosphatidylserine, at −0.5 V relative to 1 N Ag|AgCl electrode, as a function of the prothrombin concentration, at different $[Ca^{2+}]$: (□) 10^{-3} mM; (△) 0.02 mM; (●) 0.5 mM; (○) 2 mM.

capacitance does not change any more; it remains constant at a level of 6.5–7 $\mu F/cm^2$. It has been shown (Miller, 1961a,b) that every adsorbed polymer molecule contributes equally to the surface layer capacitance C^σ:

$$C^\sigma = C_L^\sigma + \theta(C_P^\sigma - C_L^\sigma) \quad (2)$$

where C_L^σ and C_P^σ are the capacitances of the intact lipid and of the protein penetrated domain, respectively, and θ is the penetrated layer fraction. In eq 2 the lipid–protein boundary effects are neglected, and its validity improves with a cooperative increase in the rise of the protein domains. θ reaches a maximal value at a surface concentration of about 1.5×10^{-12} mol/cm², which corresponds to an area of about 11 000 Å²/molecule. To estimate the degree of penetration θ, one has to know C_P^σ. Assuming that C_P^σ is equal to the specific capacitance of a pure protein monolayer at this potential (which is about 14 $\mu F/cm^2$) and that $C_L^\sigma = 1.5$ $\mu F/cm^2$, the limiting value of θ is ~0.4. This value is in keeping with the picture that the ellipsoid molecule, having a longitudinal cross-sectional area of about 4500 Å², lies flat on the surface. At the same time, conformational changes allowing larger area occupation and preventing penetration of subsequently adsorbing protein molecules cannot be excluded. In any event, penetration here is not to be taken literally but rather as a perturbation of the monolayer continuity.

The effect of prothrombin on the capacitance of the surface layer depends also on the lipid composition. The penetration of prothrombin into a monolayer containing 75% phosphatidylcholine and 25% phosphatidylserine, as inferred from the increase in capacitance, is considerably smaller than into a pure phosphatidylserine monolayer. As is evident from Figure 3, no effect on capacitance of the 25% phosphatidylserine monolayer is observed at 10^{-3} mM Ca^{2+}. At the higher concentrations of Ca^{2+}, the effect starts at higher prothrombin concentrations than on the pure phosphatidylserine monolayer. However, the limiting capacitances reached at high prothrombin concentration are about the same. The overall shape of the capacitance vs. concentration curves indicates a cooperative increase in capacitance. The effect of Ca^{2+} concentration on the capacitance of monolayers of pure phosphatidylserine and of 25% phosphatidylserine + 75% phosphatidylcholine in the presence of two concentrations (1.2 and 2.4 μg/mL) of prothrombin in the aqueous solution is presented in Figure 4. Note that on both monolayers, at sufficiently high prothrombin and Ca^{2+} concentrations, the limiting capacitance of about 7 $\mu F/cm^2$ is eventually reached. These high capacitances are obtained instantaneously upon nondamaging contact of the monolayer by the electrode. The spontaneous repair of the occasionally damaged interacting monolayers may take up to a few minutes.

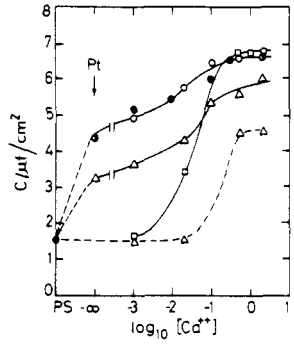

FIGURE 4: Differential capacity of condensed monolayer containing phosphatidylserine (○–○, △–△) or 75% phosphatidylcholine + 25% phosphatidylserine (□, △---△) at −0.5 V relative to 1 N Ag|AgCl electrode, as a function of $[Ca^{2+}]$ (mM) at two prothrombin concentrations: (△) 1.2 μg/mL; (○, ●, □) 2.4 μg/mL.

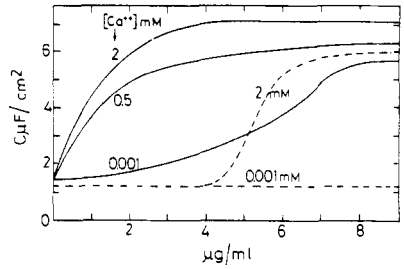

FIGURE 5: Differential capacity of condensed phosphatidylserine monolayer at −0.5 V relative to 1 N Ag|AgCl electrode, as a function of fragment I concentration at different Ca^{2+} concentrations: (solid lines) phosphatidylserine monolayer; (dashed lines) 25% phosphatidylserine + 75% phosphatidylcholine.

(*b*) *Fragment I.* Similarly to prothrombin, fragment I increased the capacitance of phosphatidylserine-containing monolayers, also giving rise to a pseudocapacitance peak. The effect increases with Ca^{2+} and with fragment I concentration and it is larger on a monolayer of pure phosphatidylserine than on a mixed monolayer of phosphatidylserine and phosphatidylcholine. The variation of the capacitance of monolayers containing only phosphatidylserine or 75% phosphatidylcholine + 25% phosphatidylserine with the concentration of injected fragment I in the subphase at different concentrations of Ca^{2+} is given in Figure 5. The capacitance of the phosphatidylserine monolayer at 0.5 mM and 2 mM Ca^{2+} increases quite steeply upon addition of relatively low concentrations of fragment I until, above 3 μg/mL, saturation values seem to be approached. At 10^{-3} mM Ca^{2+} the increase in capacitance is small, below 4 μg/mL, and it becomes steepest around 6.5 μg/mL, reaching

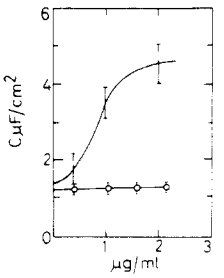

FIGURE 6: Differential capacity of condensed monolayer containing phosphatidylserine at 10^{-3} mM Ca^{2+} and 2 mM Ca^{2+} (I) or containing 75% phosphatidylcholine + 25% phosphatidylserine at 2 mM Ca^{2+} (Φ) as a function of fragment II concentration, at −0.5 V relative to 1 N Ag|AgCl electrode.

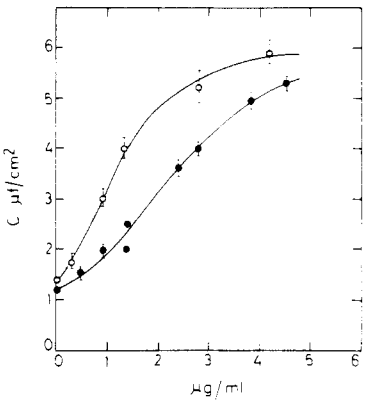

FIGURE 7: Differential capacity of condensed monolayer containing phosphatidylserine at 10^{-3} mM Ca^{2+} and 2 mM Ca^{2+} (○) or 75% phosphatidylcholine + 25% phosphatidylserine at 2 mM Ca^{2+} (●) as a function of thrombin concentration, at −0.5 V relative to 1 N Ag|AgCl electrode.

saturation values above 8 μg/mL. The sigmoidal curve obtained at this low Ca^{2+} concentration indicates a cooperative effect on capacitance. This type of cooperative phenomenon is observed also at 2 mM Ca^{2+} on the mixed monolayers containing 25% phosphatidylserine. In this respect the behavior is similar to that of prothrombin, except that higher concentrations of fragment I are required in order to penetrate the lipid layer.

(*c*) *Fragment II and Thrombin.* These two fragments do not contain γ-carboxyglutamic acid residues and cannot attach to the negatively charged monolayers by Ca^{2+} bridges. Indeed, the capacitance dependences on concentrations of fragment II and of thrombin (Figures 6 and 7) remain practically the same between 0 and 2 mM Ca^{2+}. There is, however, a dependence on the monolayer composition. In the case of thrombin, higher concentrations are required for increasing the capacitance of the mixed monolayers of 25% phosphatidylserine and 75% phosphatidylcholine than of the pure phosphatidylserine monolayer. Fragment II does not seem to affect the mixed monolayer at all. Phosphatidylcholine monolayers are known to be less penetrable than phosphatidylserine monolayers (Miller et al., 1976; Miller & Rishpon, 1977), and the present results are in keeping with this property of these monolayers. Moreover, we are witnessing here the distinctly larger tendency of thrombin to penetrate rather than fragment II. This suggests that the prethrombin fragment is probably responsible for penetration of the prothrombin into the lipid layers.

(*2*) *Pseudocapacitance Peak.* Reduction or oxidation of adsorbed electroactive groups may give rise to a pseudocapacitance peak when applying varying dc potentials or an ac modulation on slowly scanned dc potentials. The oxidation

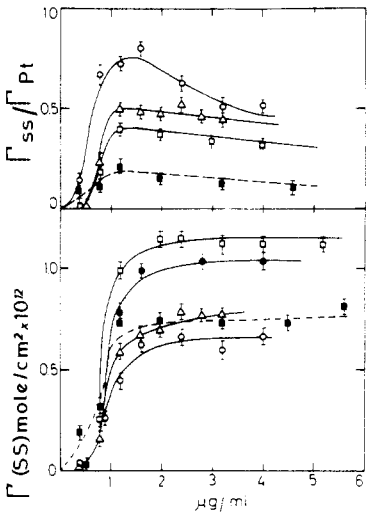

FIGURE 8: Charge transfer between cystine residues of prothrombin and the mercury electrode across phosphatidylserine-containing phospholipid monolayer, at different Ca^{2+} concentrations: (■) 2 mM; (□) 0.5 mM; (●) 0.3 mM; (△) 0.1 mM; (○) 10^{-3} mM. Lower set of curves: number of moles of cystine per square centimeter undergoing redox reaction at 80 Hz inferred from the pseudocapacitance peak area, as a function of prothrombin concentration. Upper set of curves: number of moles of reacting cystine groups per adsorbed prothrombin, as a function of prothrombin concentration.

state of the adsorbed electroactive group depends on the potential of the adsorbing electrode surface rather than on the oxidation state in the solution. When a dc potential sweep is applied in the negative potential direction, a reduction pseudocapacitance peak at the reduction potential is observed, while an oxidation pseudocapacitance peak appears when the sweep is in the opposite direction and it passes the oxidation potential. If the redox reaction is reversible, and the electroactive groups are adsorbed, the oxidation and the reduction pseudocapacitance peaks appear at the same potential.

(*a*) *Pseudocapacitance by Ac Modulation.* Pseudocapacitance peaks obtained by ac polarography of prothrombin are shown in Figure 1. The pseudocapacitance is proportional to the number of groups participating in the redox reaction. At surface saturation, the number of these groups increased with the exposure of the monolayer to the positively polarized electrode until after 1 to 2 min a constant maximal value is reached, while the final capacitance value is obtained immediately upon contact. The rate of evolution of the pseudocapacitance peak decreases with increasing concentration of Ca^{2+}. The peaks first develop slowly; then the rate picks up until after 1 to 3 min it gradually reaches its final size, producing curves of sigmoidal shape. Not all the electroactive groups with access to the electrode surface oxidize or reduce instantaneously with the variation of potential to the equilibrium state. If the electrode process is delayed either by slow electron transfer or by slow adjustment of the reacting process, this number is smaller than the number of adsorbed residues, and it depends on the frequency of the modulating ac potential.

The number of cystine residues undergoing redox reaction at the given frequency, e.g., 80 Hz in the present case, is obtained from the charge transferred, as calculated by the integral $\int C\,dV$ over the pseudocapacitance peak. Two electrons are required for reduction of a cystine residue to two cysteines. In Figure 8 the calculated number of cystine residues undergoing redox reaction at an imposed 80-Hz ac potential is plotted against the concentration of prothrombin for different concentrations of Ca^{2+}. The number of cystine residues undergoing the electrode process increases with

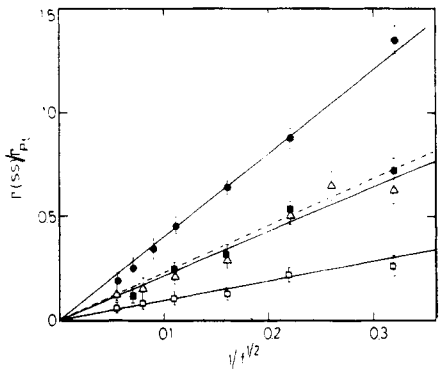

FIGURE 9: Number of moles of reacting cystine groups at 80 Hz per adsorbed prothrombin at surface saturation, as a function of the reciprocal of the square root of the frequency, at different Ca^{2+} concentrations: (●) 10^{-3} mM; (△) 0.5 mM; (□) 2 mM for pure phosphatidylserine monolayer; (■) 2 mM for 75% phosphatidylcholine + 25% phosphatidylserine.

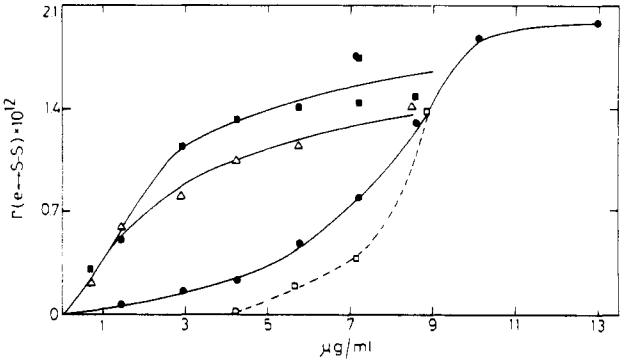

FIGURE 10: Charge transfer between cystine residues of fragment I and the mercury electrode across phospholipid monolayer. Number of moles of cystine per square centimeter undergoing redox reaction at 80 Hz as a function of fragment I concentration, at different Ca^{2+} concentrations: (■) 2 mM; (△) 0.5 mM; (●) 10^{-3} mM for pure phosphatidylserine; (□) 2 mM for 75% phosphatidylcholine + 25% phosphatidylserine.

prothrombin concentration in cooperative fashion. Unlike capacitance, at −0.5 V (Figure 2) the size of the pseudocapacitance peak reaches saturation at the same prothrombin concentration, at different concentrations of Ca^{2+}. Above a concentration of 1.5 μg/mL, a saturation value of the pseudocapacitance peak was reached in all the Ca^{2+} concentration regions investigated. As evident from Figure 8, the number of cystine residues undergoing electrode reaction as obtained from the pseudocapacitance peak first increases with Ca^{2+} concentration, together with the total surface concentration of prothrombin, but then decreases at higher concentrations of Ca^{2+}. Since the surface concentration of prothrombin has been determined independently from the increase of surface radioactivity (Lecompte and Miller, 1980), the number of cystines per prothrombin molecule undergoing redox reaction on the mercury electrode could also be calculated. The results are presented in the upper part of Figure 8. The number of cystines per prothrombin molecule contributing to the pseudocapacitance peak decreases, after the steep initial rise, with surface concentration, but the number decreases much more strongly with increases in Ca^{2+} concentration.

The calculated results presented in the upper part of Figure 8 show that even at low concentrations of Ca^{2+} only a small average fraction, less than 1 cystine residue out of 12, undergoes redox reaction on the mercury electrode under these conditions. It is evident that the electrode process is a kinetic one, probably depending on the freedom of short-range movement and of access of cystine within their molecular domains to the mercury electrode. The number of cystine residues reacting on the mercury electrode surface following the ac potential changes is expected to increase with decreasing ac frequency. The frequency dependence of the pseudocapacitance due to cystine–cysteine oxyreduction in adsorbed layers of proteins has been treated theoretically (Miller, 1971) and determined experimentally (Pavlovic & Miller, 1971). The general conclusion was that at high frequencies, when the electrodiffusion processes are far from equilibrium, the pseudocapacitance peak varies linearly with the reciprocal of the square root of the frequency ($1/f^{1/2}$). At low frequencies, equilibrium pseudocapacitance, with all the available electroactive residues following the ac potential variations and participating in the electrode process, can be approached.

In Figure 9 the number of cystine residues participating in the redox reaction is plotted against $1/f^{1/2}$ for three different concentrations of Ca^{2+}. Perfectly straight lines are obtained down to 10 Hz with no indication that equilibrium capacitance values are approached. Indeed, even at these low frequencies, only about 1 or less of 12 cystine residues per prothrombin molecule participates in the electrode process. This active cystine fraction decreases with Ca^{2+} concentration but increases when the charge density (number of phosphatidylserines) in the lipid monolayer is decreased. It is highest at 25% phosphatidylserines, which is the most active composition in the prothrombin–thrombin transformation. The tertiary structure keeps a large part of the cystine groups screened, and they are not available for reduction even during relatively lengthy exposure to the reduction potential as in dc polarography (Cecil & Weitzmann, 1964). In the present case, besides the conformational constraints of the cystine residues in proteins, additional restrictions resulting from the interaction with the lipid layer should be considered. Not all the residues in the electrode surface domain may respond to the ac modulation, but all of them should be revealed in the reduction pseudocapacitance peak during the cathodic voltametric sweep.

The dependence of the pseudocapacitance peak on fragment I concentration shows a behavior similar to that of prothrombin. As shown in Figure 10, whenever the pseudocapacitance peaks do not develop at extremely low concentrations, the dependence of the number of S–S groups participating in the electrode process on concentration shows a sigmoidal shape. The steep increase of the size of the pseudocapacitance peak across phosphatidylserine monolayers at low concentrations of fragment I at 0.5 and 2 mM Ca^{2+} may still be of sigmoidal shape with a maximal slope at indiscernibly low concentrations.

The number of cystines per adsorbed molecule undergoing the electrode process is, in fragment I, even more strongly dependent on Ca^{2+} concentration than in prothrombin. It is evident from Figure 11 that at 10^{-3} mM Ca^{2+} almost all the cystine residues can be reduced during the cathodic sweep, while less than one-third can be reduced at 10^{-1} mM Ca^{2+} and less than 5% around 2 mM Ca^{2+}. The number of residues participating in electron exchange with the electrode surface during ac potential modulation at 80 Hz is only about one-third of those reduced during the cathodic voltametric sweep. At 2 mM Ca^{2+} the number of cystines reduced per adsorbed fragment I as obtained from the pseudocapacitance peaks on monolayers containing 25% phosphatidylserine is significantly larger than on pure phosphatidylserine monolayers.

The frequency dependence of the pseudocapacitance peaks of the three fragments is similar to that of prothrombin. It varies linearly with the reciprocal of the square root of frequency between 10 and 1000 Hz.

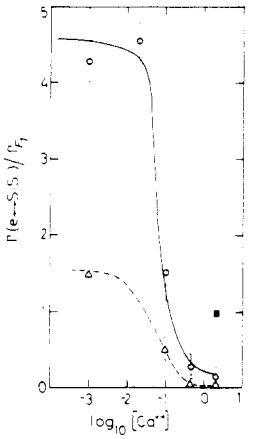

FIGURE 11: Number of moles of cystine residues, undergoing redox reaction by cyclic voltametry (○, ■) or at 80 Hz (△), per adsorbed fragment I molecule, through a phospholipid monolayer containing phosphatidylserine (○, △) or 25% phosphatidylserine (■), at surface saturation of fragment I, as a function of [Ca^{2+}] (mM).

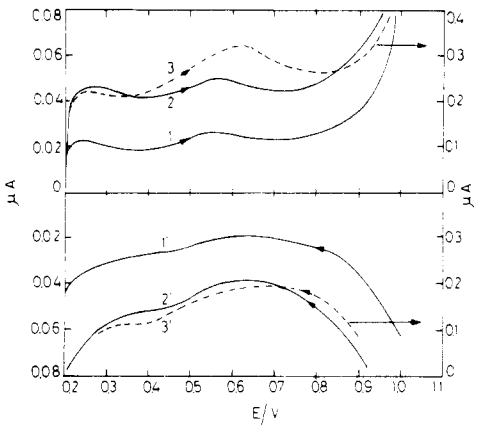

FIGURE 12: Cyclic voltammograms at pH 7.8 and 37 °C of prothrombin (0.8 μg/mL) interacting with phosphatidylserine at 0.5 mM Ca^{2+}. Current sensitivity: ordinate. Sweep rates: go (1) 0.1 V/s; (2) 0.2 V/s; (3) 1 V/s; and back 1′2′3′.

(*b*) *Pseudocapacitance by Cyclic Voltametry*. In Figure 12, cyclic voltammograms obtained on a phosphatidylserine monolayer interacting with prothrombin at 0.5 mM Ca^{2+} at three different sweep rates are presented. The reduction pseudocapacitance peak currents are proportional to the potential sweep rate, which means that the pseudocapacitance values are independent of the sweep rate except for the small shift in the potential maximum. The integral $\int i\,dt$ over the pseudocapacitance current peak, which gives the number of charges transferred to reduce the available cystine residues, is therefore independent of sweep rate. However, only a relatively small fraction of the total cystine residues on the adsorbed prothrombin molecules is available for reduction on the electrode. This fraction never exceeded 4 out of the 12 cystine residues on the adsorbed molecule in spite of the exposure of the interacting monolayer to the mercury electrode at positive polarizations, at which cystine tends to adsorb on the electrode. At low surface concentrations of prothrombin, the number of cystines reduced per adsorbed molecule is nearly constant, varying between three or four cystines per prothrombin at low Ca^{2+} concentrations (<0.1 mM) and about two cystines per molecule at 2 mM Ca^{2+}. Above a surface concentration of about 1.5×10^{-12} mol/cm^2, a limiting number of cystine groups per square centimeter is reduced. It amounts to $5–6 \times 10^{-12}$ mol/cm^2 at low Ca^{2+} concentrations and to about $3–4 \times 10^{-12}$ mol/cm^2 in the presence of 2 mM Ca^{2+}.

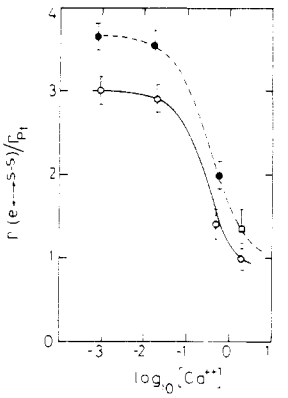

FIGURE 13: Number of moles of cystine residues undergoing redox reaction by cyclic voltametry per adsorbed prothrombin molecule, on a phospholipid monolayer at surface saturation of prothrombin for a pure phosphatidylserine monolayer (○), for 75% phosphatidylcholine + 25% phosphatidylserine (□), and at a prothrombin concentration of 0.8 μg/mL (●) as a function of [Ca^{2+}] (mM).

Any further adsorption does not contribute to the pseudocapacitance peak.

In Figure 13 the dependence of the number of cystines reduced per adsorbed prothrombin molecule is plotted against log [Ca^{2+}] for different prothrombin concentrations in the aqueous solution. The pronounced change in the ratio between 0.1 and 0.5 mM Ca^{2+} suggests a conformational change in this region brought about by the lipid–Ca^{2+}–proton bonds. However, it is also possible that the additionally adsorbed molecules, at the higher Ca^{2+} concentrations, simply do not penetrate.

The cathodic pseudocapacitance peak is practically independent of the starting potential at potentials positive of the peak potential. No pseudocapacitance peak is obtained when the starting potential was more negative than the peak potential, e.g., −0.8 V; however, the capacitance increased, indicating overall protein penetration. If the monolayer was first exposed to positive polarization, e.g., −0.3 V for a few minutes, and then transferred to −0.8 V from where the potential scan started in the positive direction, the pseudocapacitance peak was observed. It diminished, however, with exposure time at the negative potential until it disappeared completely after 10–15 min.

The cysteine residues formed by reduction during the sweep toward negative polarization tend to desorb, and when the sweep is reversed they have to diffuse back to the electrode surface through the lipid layer in order to be reoxidized. The oxidation pseudocapacitance peak is therefore shallower than the reduction peak and shifted to more positive potentials, and the shift increases with the sweep rate.

In Figures 14 and 15, the number of moles of cystine of fragment II and thrombin, respectively, participating in the electrode process through the lipid monolayers is given. The number of cystines of fragment II reduced during the cathodic sweep of cyclic voltametry or exchanging electron through phosphatidylserine monolayers during ac potential modulation is about three times larger than that of thrombin. The number of cystine residues of thrombin participating in the electrode process through the mixed monolayers of 75% phosphatidylcholine + 25% phosphatidylserine is, to some extent, smaller than that reacting through the pure phosphatidylserine monolayer. At the same time, the mixed monolayer completely impeded the electrode reaction of the cystine residues of fragment II. The larger capacitance peaks of fragment II through the phosphatidylserine monolayer are in keeping with its larger cysteine content (5.6% of the total amino acids) as compared with thrombin (2.6% of the total amino acids),

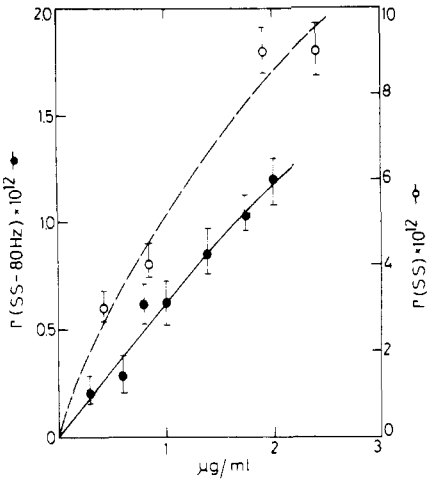

FIGURE 14: Number of moles of cystine residues of fragment II per square centimeter undergoing redox reaction at 80 Hz (●) or by cyclic voltametry (○) through a phosphatidylserine monolayer on the electrode, at 10^{-3} and 2 mM Ca^{2+} as a function of fragment II concentration.

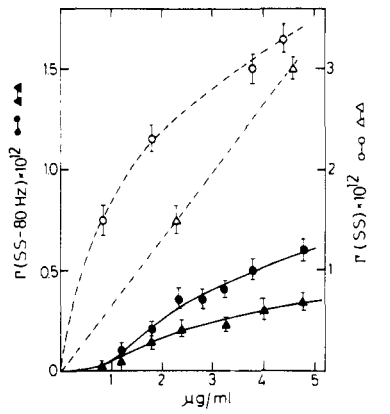

FIGURE 15: Number of moles of cystine residues of thrombin per square centimeter undergoing redox reaction at 80 Hz (●, ▲) or by cyclic voltametry (○, △) through condensed monolayer: (○, ●) 100% phosphatidylserine at 2 and 10^{-3} mM Ca^{2+}; (△, ▲) 25% phosphatidylserine at 2 mM Ca^{2+} as a function of thrombin concentration.

provided that the two proteins permeate the monolayer equally well. This seems to be the case in the pure phosphatidylserine monolayer in spite of the negative net charge of fragment II. It is, therefore, surprising that Ca^{2+} up to 2 mM does not seem to enhance their interaction, as it does not have any effect either on the increase of capacitance or on the rise of the pseudocapacitance peak. Presumably it affects very little the charge densities at these concentrations, but it might have an effect at higher concentrations.

The hydrophobicity of fragment II is, however, lower than that of thrombin, which may explain, at least in part, why the latter penetrates the mixed monolayer containing 75% phosphatidylcholine while the first one does not. The positive net charge of thrombin may also contribute to the penetration of the distortion of the mixed monolayer containing 25% negatively charged lipids.

Discussion and Conclusion

Experiments described in this series of papers confirm the conclusions of previous publications, based on work with liposomes and the bilayer vesicles (Nelsestuen & Lim, 1977; Dombrose et al., 1979), that prothrombin adsorbs on phosphatidylserine-containing lipid layers. We obtained, however, 30 times larger binding constants both for prothrombin and for its fragment I. The difference may stem, in part, from the fact that we worked in lower concentration regions. There are indications, both in our work and in carefully examined published results on bilayer vesicles (Nelsestuen & Lim, 1977; Dombrose et al., 1979), that there may be two different binding constants, a larger one in the very low concentration region and a smaller one at increased concentrations. It seems, however, that an important part of the difference results from the planarity of the monolayer surface in contradistinction to the highly curved vesicle surface, which affects the packing of the head groups and thus affects electrostatic and possibly hydrophobic interactions. Prothrombin does not just bind to the monolayer by Ca^{2+} bridges; appreciable binding also occurs in the absence of Ca^{2+}.

The aim of this work was to obtain some information on the structure of the interaction product between phospholipid monolayers containing phosphatidylserine and prothrombin and on their interaction dynamics. One can question the biological relevance of these experiments or even their significance with respect to interactions and biological activity on lipid bilayer membranes, especially since the measurements were carried out on monolayers in contact with a mercury electrode surface. The interface between the polar plane of a monolayer and an aqueous solution is certainly equivalent to that between a polar plane of a bilayer and an aqueous phase of equal composition. Thus, as long as the interactions are only in the polar region, there should not be any appreciable differences.

There may be, however, differences between planar surfaces of monolayers or of planar bilayer sections in membranes and between highly curved surfaces in small vesicles. These differences may even affect the tendency of an interacting protein to penetrate the hydrocarbon layer. Penetration of the hydrocarbon layer does not necessarily mean that a pure protein–hydrocarbon interface is formed. The lipid polar groups may accompany at least in part the penetrating protein molecules, thus perturbing the continuity of the polar and the hydrocarbon planes. By this mechanism, penetration of a monolayer or of one layer of a bilayer is substantially more probable than complete spanning of a bilayer. The tendency to penetrate will also be affected by the possible interactions on the other side of a single layer. In this respect, monolayers on different supports may differ from each other and from one lipid layer in a bilayer. Half a bilayer could be, in this sense, most equal to a monolayer at a water–hydrocarbon interface. However, the air surface and the mercury surface, when not highly polarized (less than 0.5 V from the zero charge point), are highly hydrophobic surfaces, and amphipathic molecules like phospholipids orient with their hydrophobic hydrocarbon chains toward them. The energy requirement for replacement of a hydrocarbon residue by a polar amino acid residue is about the same at the three interfaces: between two hydrocarbon planes, between a hydrocarbon plane of a lipid layer and air, or mercury at zero charge. Charging the mercury surface creates a field across the lipid layer that enhances penetration or displacement of nonpolar hydrocarbon chains by more polar residues. However, there may also be more specific interaction between the mercury surface and certain groups., e.g., cystine at positive polarization of the mercury surface.

The overall increase in capacitance was independent of the starting potential to which the monolayer had been exposed for different lengths of time. One can assume, therefore, that prothrombin penetrates or perturbs the lipid monolayer irrespective of the supporting surface. At the air–water interface such a penetration would probably affect the rate of water evaporation through the monolayer. Conductance, capaci-

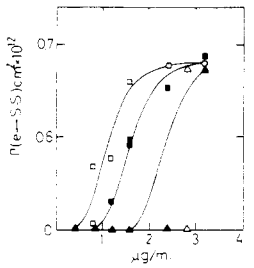

FIGURE 16: Number of moles of cystine residues of prothrombin per square centimeter undergoing redox reaction at 80 Hz, inferred from the pseudocapacitance peak area, through a monolayer containing 75% phosphatidylcholine + 25% phosphatidylserine, at different Ca^{2+} concentrations: (■) 2 mM; (□) 0.5 mM; (▲) 0.02 mM; as a function of prothrombin concentration.

tance, and transport through bilayers might also be affected by prothrombin.

In the case of prothrombin and of fragment I when surface concentration was measured in parallel with capacitance, it was shown that capacitance reaches saturation values, while the overall adsorption continues. It was concluded from this that the surface conformation and the orientation of the molecules adsorbed up to a certain critical surface concentration are spread out, revealing hydrophobic residues which thus contribute to the free energy of adsorption and perturb and penetrate the lipid layer. The subsequent molecules are then attracted to the negatively charged lipid layer via Ca^2 bridges from their γ-carboxyglutamic acid residues. The conformation of fragment I, in solution, depends on Ca^{2+} concentration (Prendergast & Mann, 1977). A conformational change of prothrombin with Ca^{2+} concentration has been also postulated (Nelsestuen, 1976). The dependence of the number of cystine residues per prothrombin molecule exposed to electrode process may support this view. Prothrombin preserves its native conformation when interacting with a phospholipid surface, but when attached to it at low concentrations it may orient with its long axis parallel to the surface. Thus, hydrophobic and electrostatic interactions between the relatively more hydrophobic and positively charged prothrombin and the negatively charged lipid layer may take place. At the same time, if Ca^{2+} is present, Ca^{2+} bridges between fragment I and the negatively charged phospholipid surface can be maintained. The fact that thrombin but not fragment II penetrates the monolayer containing 75% phosphatidylcholine is in keeping with the picture. One has, of course, always to bear in mind that, after their cleavage, the different fragments may change their conformation, and any evidence derived from the behavior of the separated fragments is to be considered cautiously.

Another interesting feature is the dynamic behavior of the interacting proteins. No pseudocapacitance peak is obtained when the potential sweep is applied immediately upon contact of the monolayer by the mercury electrode, even though penetration has occurred at the air–water interface and capacitance increase is instantaneous. The size of the pseudocapacitance peak increases with exposure time until it is fully developed after 1 to 3 min, depending on the concentration of Ca^{2+}. It seems, therefore, that the cystine residues are not among the amino acid residues attracted to the air–monolayer interface. When, upon exposure, it binds to the positively polarized mercury surface, a relatively slow rearrangement occurs, allowing access of the cystine residues of the surface. If the monolayer is first exposed to a positively polarized mercury surface (e.g., −0.3 V), the potential is changed to, e.g., −0.8 V, and then the potential sweep is applied after different exposure times, a disappearance of the pseudocapacitance peak is observed within a few minutes. The relevance of the slow ($\tau \simeq$ minutes) Ca^{2+}-dependent, relatively long-range movement of sections of interacting molecules is not yet clear. However, any exchange or displacement by other molecules would have a similar time course. Displacement from the surface is an essential step in any reaction taking place in the surface with products being eluted and transferred to other parts of the system.

There is a quantitative and qualitative dependence of the capacitance and of the pseudocapacitance peaks on lipid layer composition. A distinct feature, which becomes apparent at monolayers containing 25% phosphatidylserine, is the cooperative dependence of the capacitance and of the pseudocapacitance peaks on the prothrombin concentration. This does not mean that the cooperativity is absent on the pure phosphatidylserine monolayer; it appears, but at extremely low prothrombin concentrations, and is hardly discernible. An example of cooperative development of the pseudocapacitance peak on a monolayer containing 25% phosphatidylserine and 75% phosphatidylcholine is given in Figure 16. This figure suggests that adsorption and cooperative penetration of the adsorbed prothrombin molecules are responsible for the size of the pseudocapacitance peaks.

In general, Ca^{2+} causes less stiff binding of prothrombin and of fragment I to monolayers containing only 25% phosphatidylserine than to pure phosphatidylserine monolayers, allowing more freedom of movement. This more pliable binding may be responsible for the maximal catalytic activity of this lipid composition in the prothrombin–thrombin transformation.

Acknowledgments

We thank Dr. J. Elion and Dr. R. Benarous for the purified prothrombin and its fragments.

References

Bloom, J. W., & Mann, K. G. (1979) *Biochemistry 18*, 1957–1961.

Cecil, R., & Weitzmann, P. D. J. (1964) *Biochem. J. 93*, 1–11.

Dombrose, F. A., Gitel, S. N., Zawalich, K., & Jackson, C. M. (1979) *J. Biol. Chem. 254*, 5027–5040.

Kolthoff, I. M., & Barnum, C. (1941) *J. Am. Chem. Soc. 63*, 520–528.

Lecompte, M. F., & Miller, I. R. (1980) *Adv. Chem. Ser.* (in press).

Lecompte, M. F., Miller, I. R. Elion J., & Benarous, R. (1980) (preceding paper in this issue).

Miller, I. R. (1961a) *Trans. Faraday Soc. 57*, 301–311.

Miller, I. R. (1961b) *J. Mol. Biol. 3*, 229–240, 357–361.

Miller, I. R. (1971) *Experientia, Suppl. 18*, 477–487.

Miller, I. R., & Rishpon, J. (1977) in *Electrical Phenomena at the Biological Membrane Level* (Roux, E., Ed.) pp 93–110, Elsevier, Amsterdam.

Miller, I. R., Rishpon, J., & Tenenbaum, A. (1976) *Bioelectrochem. Bioenerg. 3*, 528–542.

Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648–5656.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164–4171.

Pagano, R. E., & Miller, I. R. (1973) *J. Colloid Interface Sci. 45*, 126–137.

Pavlovic O., & Miller I. R. (1971) *Experientia, Suppl. 18*, 513–524.

Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.

Rishpon, J., & Miller, I. R. (1977) *J. Colloid Interface Sci. 60*, 406–407.