Phosphatidylserine-Containing Membranes Alter the Thermal Stability of Prothrombin's Catalytic Domain: A Differential Scanning Calorimetric Study†

Barry R. Lentz,* Chao-Ming Zhou, and Jogin R. Wu

Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260

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ABSTRACT: Denaturation profiles of bovine prothrombin and its isolated fragments were examined in the presence of Na_2EDTA, 5 mM $CaCl_2$, and $CaCl_2$ plus membranes containing 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) in combination with bovine brain phosphatidylserine (PS). We have shown previously [Lentz, B. R., Wu, J. R., Sorrentino, A. M., & Carleton, J. A. (1991) *Biophys. J. 60*, 70] that binding to PS/POPC (25/75) large unilamellar vesicles resulted in an enthalpy loss in the main endotherm of prothrombin denaturation ($T_m \sim 57$–58 °C) and a comparable enthalpy gain in a minor endotherm ($T_m \sim 59$ °C) accompanying an upward shift in peak temperature ($T_m \sim 73$ °C). This minor endotherm was also responsive to Ca^{2+} binding and, in the absence of PS/POPC membranes, corresponded to melting of the N-terminal, Ca^{2+} and membrane binding domain (fragment 1). Peak deconvolution analysis of the prothrombin denaturation profile and extensive studies of the denaturation of isolated prothrombin domains in the presence and absence of PS/POPC vesicles suggested that membrane binding induced changes in the C-terminal catalytic domain of prothrombin (prethrombin 2) and in a domain that links fragment 1 with the catalytic domain (fragment 2). Specifically, the results have confirmed that the fragment 2 domain interacts with and stabilizes the prethrombin 2 domain and also have shown that fragment 2 interacts directly with the membrane. In addition, the results have demonstrated a heretofore unrecognized interaction between the catalytic and membrane binding domains. This interaction can account for another portion of the denaturation enthalpy that appears at high temperatures in the presence of membranes. Our data lead to the hypothesis that membrane-specific alterations in interdomain interactions and domain stability may account for the observation that the C-terminal catalytic domain of prothrombin is more effectively proteolyzed to thrombin on PS-containing membranes as compared to other membrane surfaces [Pei, G., Powers, D. D., & Lentz, B. R. (1993) *J. Biol. Chem. 268*, 3226].

The prothrombinase complex, which consists of a serine protease (factor Xa), a protein cofactor (factor Va), Ca^{2+}, and an appropriate acidic lipid membrane surface, plays the key role in blood coagulation of converting prothrombin to thrombin (Zwaal & Hemker, 1982; Mann, 1987). Because prothrombin is efficiently converted to thrombin only by the membrane-bound prothrombinase in the presence of Ca^{2+}, much effort has gone into characterizing the interactions of prothrombin with Ca^{2+} and with acidic lipid membranes. The N-terminal third of prothrombin (*fragment 1*, F1,[1] 22 kDa) contains roughly 10 γ-carboxylated glutamic acid residues which are thought to mediate prothrombin binding to acidic lipid membranes *via* a Ca^{2+}-bridging mechanism (Dombrose *et al.*, 1979). Intrinsic fluorescence quenching experiments (Nelsestuen, 1976; Prendergast & Mann, 1977), circular dichroism experiments (Bloom & Mann, 1978; Marsh *et al.*, 1979), antibody binding experiments (Borowski *et al.*, 1986), and differential scanning calorimetric (DSC)[1] studies (Ploplis *et al.*, 1981; Lentz *et al.*, 1991) all have indicated that F1 undergoes one or two Ca^{2+}-induced conformational changes. The Ca^{2+}-induced conformational change in the F1 domain is necessary for binding to acidic lipid membranes (Jackson *et al.*, 1975; Nelsestuen, 1976). The F1 domain of prothrombin is generally viewed as providing the membrane binding function of the molecule. However, evidence exists that membrane binding of whole prothrombin and membrane binding of its N-terminal F1 domain are not identical (Tendian *et al.*, 1991; Pearce *et al.*, 1993).

After proteolytic removal of the F1 domain by thrombin cleavage, what remains of prothrombin is termed *prethrombin 1* (Pre 1)[1]. The N-terminal 118 amino acid residues of this peptide are proteolytically freed by factor Xa and are termed *fragment 2* (F2,[1] 12.5 kDa). Like F1, this contains a kringle loop, although this kringle has a structure different from F1 and other kringles that allows F2 to assume a conformation that optimizes its interaction with the catalytic domain of prothrombin (Arni *et al.*, 1993). The function of this domain is unknown, although it is evident that it acts as a *linker domain* between the *membrane binding region* (F1) and the remaining (C-terminal) portion of prothrombin, termed *prethrombin 2* (Pre2,[1] 38 kDa). This region is often termed the *catalytic domain*, since it becomes *thrombin*. The F2 region binds tightly to Pre2 and thrombin (Myrmel *et al.*, 1976) *via* an ionic interaction (Arni *et al.*, 1993). The peptide bond between F2 and Pre2 (Arg^{274}–Thr^{275}) is one of the two cut by factor Xa during activation of prothrombin to thrombin (Mann, 1976). The other bond cut by factor Xa (Arg^{323}–Ile^{324}) is within the Pre2 region; after this is cut, the two pieces of the resulting thrombin are held together by a disulfide

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[1] Abbreviations: DSC, differential scanning calorimetry; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; PS, bovine brain phosphatidylserine; LUV, large, unilamellar vesicle(s); DEAE-cellulose, (diethylaminoethyl)cellulose; MOPS, 3-(*N*-morpholino)propanesulfonic acid; S-2238, phenylalanylpipecolylarginine-*p*-nitroaniline (synthetic substrate for thrombin); Na_2EDTA, disodium ethylenediaminetetraacetic acid; *R*, ratio of the calorimetric to van't Hoff enthalpies; F1, fragment 1 (membrane binding domain); F2, fragment 2 (linker domain); F1.2, fragment 1.2 (covalently linked F1–F2); Pre1, prethrombin 1; Pre2, prethrombin 2 (catalytic domain).

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bond. Thus, the Pre2 region and thrombin have the same molecular mass (38 kDa), although, due to the proteolysis of one peptide bond, they have different structural (Stevens & Nesheim, 1993) and functional properties: thrombin is a potent and specific serine protease while Pre2 has no detectable proteolytic activity.

Ploplis *et al.* (1981) used differential scanning calorimetry to conclude that the F1 and Pre1 regions of bovine prothrombin denatured roughly independently of each other, *i.e.*, that they behaved as independent structural domains. Addition of Ca^{2+} shifted the denaturation temperature and increased the denaturation enthalpy of the F1 domain but had little effect on the Pre1 peak, consistent with the spectroscopic evidence mentioned above for a Ca^{2+}-induced conformational shift in the F1 region of prothrombin. Since acidic lipid membranes have a specific influence on the k_{cat} of prothrombin activation (Pei *et al.*, 1993), we have attempted to determine the influence of different membranes on the structure and domain organization of prothrombin. Binding to acidic lipid membranes appears not to induce change in the F1 domain, as judged by Fourier transform infrared spectroscopy to detect secondary structural changes (Wu & Lentz, 1991). Using differential scanning calorimetry (DSC) to follow denaturation profiles, we have shown (Lentz *et al.*, 1991) that binding to phosphatidylserine (PS)[1]-containing model membranes resulted in an enthalpy loss in the main (Pre1) endotherm and a comparable enthalpy gain in the minor endotherm accompanied by an upward shift in the endotherm peak temperature ($T_m \sim 73$ °C). The present study examines in more detail the effects of membrane binding on individual prothrombin domains and on the interaction between domains. The results have shown that the F2 domain interacts not only with the Pre2 domain but also with Ca^{2+} and probably directly with the membrane. In addition, a previously undetected interaction of the F1 domain with the Pre2 domain alters Pre2 thermal stability when the F1 domain is bound to a PS-containing membrane. We may now conclude that the high-temperature endotherm observed on binding specifically to PS membranes reflects not only the shifted melting of the F2 domain but also the stabilization of a subdomain of the Pre2 domain due to its altered interaction with the membrane binding F1 domain.

MATERIALS AND METHODS

Materials. Calcium chloride was reagent grade from Fisher Chemicals. Bovine brain phosphatidylserine (PS) and 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The thrombin-specific substrate phenylalanylpipecolylarginine-*p*-nitroaniline (S-2238) was purchased from Helena Laboratories (Beaumont, TX). All other chemicals were ACS reagent grade or the best available grade; all solvents were HPLC grade.

Protein and Peptide Isolation and Handling. Bovine prothrombin was purified from $BaSO_4$ adsorbate of 12 L of bovine plasma obtained *via* centrifugation (using a commercial creme separator or a DuPont-Sorvall RC3B centrifuge, 30 min at 4000 rpm in 1-L buckets) from freshly collected bovine blood. The blood was collected into an inhibitor cocktail (10/1 v/v blood/cocktail) consisting of 0.1 M sodium oxalate, 0.1 M benzamidine hydrochloride, 0.1 mg/mL soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), and 2.5 units/mL heparin (Sigma). The $BaSO_4$ adsorption and elution procedures were largely as described by Esmon (1973), except that elution was into a buffer containing 0.1 M sodium citrate, 5 mM benzamidine hydrochloride, 0.02% NaN_3, and 5 mM Na_2EDTA at pH 5.8 (4 °C). The eluate was diluted with distilled water to 0.06 M sodium citrate and batch-adsorbed onto 21.5 mL of QAE-Sephadex (Sigma) equilibrated in 250 mM NaCl, 20 mM Tris, 5 mM benzamidine hydrochloride, and 0.02% NaN_3, pH 7.4 (4 °C). The supernatant from this batch adsorption was saved, and the QAE-Sephadex was washed first with 200 mL of the same buffer containing 200 mM NaCl and then with 200 mL of this buffer containing 250 mM NaCl. The washed QAE-Sephadex was layered onto a 110-mL QAE-Sephadex column, and this was eluted with a gradient of NaCl from 0.25 to 0.75 M to recover factor X separated from part of the prothrombin. The supernatant (diluted 1/1 with 20 mM Tris/5 mM benzamidine hydrochloride, pH 7.4) and wash from this first adsorption were batch-adsorbed to 32 mL of QAE-Sephadex equilibrated in 20 mM Tris/5 mM benzamidine hydrochloride, and this material was washed as in the first adsorption. The washed QAE-Sephadex was layered onto a 90-mL QAE column equilibrated in buffer containing 0.25 M NaCl, and this column was eluted with a 0.25–0.45 NaCl gradient. The prothrombin fractions from these two columns were combined, concentrated using Centriprep 10, Centricon 10 (Amicon, Danvers, MA), or RCF-Confilt (Bio-Molecular Dynamics, Beaverton, OR) centrifugal concentrators, and further purified on a BioGel P100 (Bio-Rad, Richmond, CA) column. At this stage, prothrombin was stored in 1 mM benzamidine/1 mM phenylmethanesulfonyl fluoride at −70 °C. A final purification step was performed 1 day prior to an experiment by HPLC on a Perkin-Elmer Isopure LC system using a Mono Q HR 5/5 ion exchange column (Pharmacia, Norwalk, CT). This step removed small quantities (<10%) of proteolysis products that occasionally formed during storage (Lentz *et al.*, 1991).

Bovine F1 and Pre1 were obtained from thrombin cleavage of purified prothrombin at Arg^{156}–Ser^{157}. For some preparations, thrombin in solution was used for the proteolysis (Heldebrant *et al.*, 1973), and for other preparations, thrombin was coupled to an activated affinity gel, Affi-Gel 10 (Bio-Rad, Rockville Centre, NY). For proteolysis with solution thrombin, 1 NIH unit of Sigma thrombin (600 NIH units/mg) was added per milligram of prothrombin and incubated at 37 °C for 3 h. After the incubation, the reaction was stopped with 0.4 μM PPACK. For proteolysis with immobilized thrombin, prothrombin was incubated with the Affi-Gel-linked thrombin for 3 h at 37 °C and then centrifuged in a tabletop centrifuge to remove the gel. F1 and Pre1 were separated by ion-exchange chromatography on a DEAE-cellulose column or a Mono Q HR 5/5 column (Pharmacia) coupled to a Perkin-Elmer Isopure LC system. F1 was further purified by gel filtration (Bio-Rad P-100) to remove any residual prothrombin. The intrinsic fluorescence of purified F1 was quenched 41–51% (λ_{ex} = 295 nm, λ_{em} = 345 nm) by 5 mM $CaCl_2$ (Nelsestuen, 1976; Prendergast & Mann, 1977; Nelsestuen *et al.*, 1981).

Bovine Pre2 and fragment 1.2 (F1.2) were generated from cleavage of prothrombin at Arg^{274}–Thr^{275} by factor Xa in the presence of the reversible thrombin inhibitor dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) as described previously (Stevens *et al.*, 1993; Tendien *et al.*, 1993).

Protein concentrations were determined by absorbance measurements using extinction coefficients at 280 nm of 1.44 mL mg^{-1} cm^{-1} for prothrombin, 1.05 mL mg^{-1} cm^{-1} for F1, 1.67 mL mg^{-1} cm^{-1} for Pre1 (Mann, 1976), 1.95 mL mg^{-1} cm^{-1} for Pre2 (Winzor & Scheraga, 1964), and 1.23 mL mg^{-1}

cm^{-1} for fragment 1.2 (Esmon & Jackson, 1974a) and corrected for light scattering with the absorbance measured at 320 nm (Donovan, 1969). Prothrombin, Pre1, and Pre2 concentrations were also confirmed by using the synthetic chromogenic substrate S2238 to assay thrombin formation (based on an active-site-titrated standard thrombin) from protein activated by *Echis carinatus* venom. Protein preparations were greater than 95% homogeneous as evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using Kodavue (Eastman Kodak Co., Rochester, NY) staining procedures.

Phospholipid Vesicles. Large unilamellar vesicles (LUV) were prepared by the method of Szoka *et al.* (1980), while extruded LUV were prepared according to the procedure of Mayer *et al.* (1986). Measured volumes of phosphate-assayed (Chen *et al.*, 1956) lipid stocks (in N_2-saturated $HCCl_3$/H_3COH, 1/1) were mixed and dried onto the walls of small, round-bottom flasks or glass culture tubes using a stream of argon. The resulting lipid film was dissolved in a small volume of cyclohexane, and the solution was frozen and lyophilized for at least 8 h to a white, anhydrous powder. To this was added an appropriate volume of buffer, and procedures were followed to prepare vesicles as described above. Vesicle compositions for most experiments were 25/75 bovine PS/POPC, with some experiments performed with 10/90 or 15/85 bovine PS/POPC to test for the effects of different surface concentrations of PS.

Differential Scanning Calorimetry. DSC measurements were performed on a Microcal (Amherst, MA) MC-2 biological microcalorimeter equipped with a Keithly 150B amplifier. Samples were deaerated 0.5 h before being loaded into the calorimeter cell with a Hamilton microliter/gastight syringe. The buffer for calorimetry scans was 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS, Sigma Chemical Co.) and 150 mM NaCl, pH 7.4. Buffer was passed over a Chelex-100 column to remove metal ions. In addition, for samples containing no Ca^{2+}, the buffer contained 0.1 mM Na_2EDTA. For samples containing calcium, the buffer contained 5 mM $CaCl_2$. Protein samples (0.2–1.0 mg/mL) were dialyzed into scan buffer just prior to a scan, and a portion of the equilibrated buffer was used in the reference cell of the calorimeter. Heat capacity profiles of membrane samples prepared in the same buffer were obtained in separate scans for subtraction from profiles of samples containing protein plus lipid. The scan rate for all experiments was 60 °C/h. No scan rate dependence of prothrombin or selected fragment thermograms was detected at or below this scan rate (20–60 °C/h). The lack of scan rate dependence argues that our observations should reflect reversible denaturation followed by slow and irreversible aggregation and precipitation (Freire *et al.*, 1990), leading overall to an irreversible denaturation process. A minimal scan rate dependence is argued to mean that denaturation profiles can be interpreted in terms of the thermodynamic conformational state of the native protein (Sánchez-Ruiz *et al.*, 1988).

Data (voltage proportional to sample heat capacity) were recorded every 5 s using a Swan AT12 (Tussey Computer Products, State College, PA) microcomputer interfaced to the calorimeter. All experiments were performed at least in duplicate to be certain that subtle effects were reproducible. Data are presented for representative experiments. Because thermograms of samples containing only lipid membranes showed no discernible thermal events in the temperature range of protein denaturation, no correction was made for a phospholipid "background." Calorimetric data were converted to heat capacity (kilocalories per mole per degrees kelvin) *versus* temperature profiles, and entire endotherms (consisting of several heat capacity peaks) were integrated using the continuous base-line algorithm (Sturtevant, 1987). Multiple peaks were resolved using software provided by Microcal, and individual peak enthalpies were obtained with the same software. These deconvolution algorithms resolve overlapping peaks according to three basic models; the least restrictive model (independent, non-two-state transitions) was used. In this case, the ratio of the integrated calorimetric enthalpy to the van't Hoff enthalpy (R) for any resolved peak can provide information about the nature of the denaturation process associated with that peak (Strurtevant, 1987). A ratio close to 1 indicates a simple, two-state, all-or-nothing denaturation event characteristic of a simple globular protein (Sturtevant, 1987). A ratio greater than 1 indicates that more than one two-state transition is associated with the resolved endotherm. A ratio significantly less than 1 can have one of several causes: (1) overestimates of protein concentrations; (2) artifacts due to aggregation; or (3) complex interactions between protein domains that lead to highly coupled and sequential unfolding events (Ramsay & Freire, 1990). The lack of any significant scan rate dependence and the use of careful protein determination methods argue against the first two possibilities for the data presented here.

RESULTS AND DISCUSSION

Summary: Model for Effects of Membranes on Prothrombin Domain Structure. In order to best present our results, we first summarize our interpretation in terms of a model for how PS-containing membranes affect the domain organization and interactions within prothrombin:

(1) An unanticipated interaction exists between the Pre2 and F1 domains. Melting of Pre2 plus F1 becomes very complex in the presence of membranes in a way that indicates that a subdomain of Pre2 is altered by the F1–membrane and F1–Pre2 interactions.

(2) F2 melting is significantly altered in the presence of Ca^{2+} and PS/POPC membranes, indicating a direct F2–membrane interaction.

(3) Binding of prothrombin to a PS-containing membrane alters both the Pre2 and F2 domains.

(4) Ca^{2+} binding to F2 alters interaction with Pre2 such that some subdomain of F2 is stabilized to melt at higher temperature, but not in intact prothrombin. The combination of effects 1, 2, and 3 can account qualitatively for the observed influence of PS-containing membranes on the thermal denaturation profiles of prothrombin.

Apparently, the Pre2 domain is altered by binding to a PS-containing membrane both through direct interaction with the F1 domain and through its known interaction with the F2 domain, which also interacts with the membrane. The influence of membrane and Ca^{2+} binding on the domain structure and interdomain interactions within bovine prothrombin is illustrated in Figure 1. Our results will be described and then discussed in the context of this model.

Denaturation of whole bovine prothrombin is illustrated by the thermograms shown in Figure 2. As reported previously (Ploplis *et al.*, 1981; Lentz *et al.*, 1991), prothrombin denaturation was described by two exotherms: a main one peaked at 58.4 °C and a minor one at 63.9 °C (see Table 1), with the major peak corresponding to Pre1 melting and the minor to F1 melting. Denaturation of prothrombin in the presence of 5 mM $CaCl_2$ reduced the enthalpy associated with the main peak and made it sharper (more cooperative,

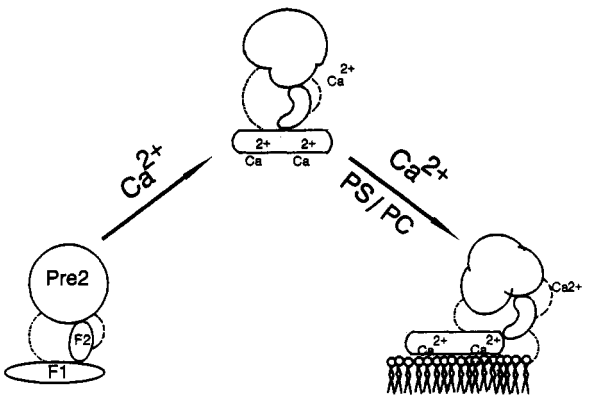

FIGURE 1: Model for the effects of Ca^{2+} and PS/POPC membranes on the conformation and interactions of prothrombin structural domains.

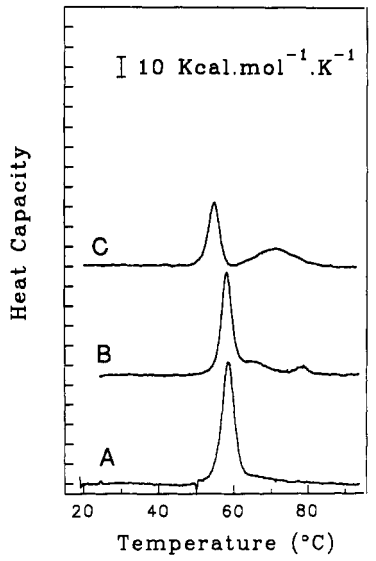

FIGURE 2: Thermal denaturation profiles of bovine prothrombin in the absence and presence of Ca^{2+} and PS/POPC (25/75) membranes. (A) Prothrombin (1.18 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (B) Prothrombin (1.95 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (C) Prothrombin (0.85 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$ and 10 mM PS/POPC LUV. The heating scan rate was 60 °C/h, although there was no significant difference between the peak positions for scans obtained at slower rates (to 20 °C/h).

$R = 0.7$) and enhanced slightly the second peak while shifting it to higher temperature. In addition, a very minor (ΔH = 18 kcal/mol) and variable endotherm was detected at 78 °C. Because it is so small and somewhat variable, this endotherm was not detected previously (Ploplis *et al.*, 1981; Lentz *et al.*, 1991). Upon binding to PS-containing membranes, the main endotherm shifted to lower temperature, the second peak shifted to higher temperature, and the high-temperature peak (peak 4 in Table 1) tripled in enthalpy. We have previously reported that this behavior was specific for PS-containing membranes and was not observed for neutral POPC membranes to which prothrombin does not bind or even for membranes that contain another acidic lipid (phosphatidylglycerol) which do bind prothrombin (Lentz *et al.*, 1991). This indicates that the observed effects do not reflect nonspecific effects of phospholipid membranes on prothrombin denaturation or nonspecific binding of prothrombin to any acidic lipid membrane.

Interaction between the Pre2 and F1 Domains Is Altered by Binding to Membranes. As has already been noted (Ploplis *et al.*, 1981), melting of the Pre2 domain alone could not be detected calorimetrically. However, in experiments on mixtures of F1 with Pre2, we have detected two distinct endotherms: one at 62 °C corresponding to the melting of F1 and one at 46 °C (Figure 3A; Table 2). The latter is presumed to represent Pre2 melting, since denaturation of the isolated Pre2 domain can be detected between 39 and 43 °C by light scattering (Tendian *et al.*, 1994). This result provides clear evidence of an unanticipated interaction between prothrombin's F1 and Pre2 domains. Upon addition of Ca^{2+}, the F1 peak was shifted to higher temperature, while the Pre2 peak broadened somewhat and increased slightly in enthalpy (Figure 3B). In the presence of PS/POPC membranes and Ca^{2+}, the Pre2 domain melting shifted to somewhat lower temperature and was resolvable into two cooperatively melting endotherms (42 and 47 °C) with a combined enthalpy roughly half that seen in the absence of membranes (Figure 3C; Table 2). Enthalpy roughly equivalent to that missing from the Pre2 endotherm appeared as a shoulder on the high-temperature edge of the F1 peak (Figure 3; Table 2). This is qualitatively very similar to the behavior seen for whole prothrombin, except that the high-temperature peak does not match exactly the membrane-induced high-temperature peak seen for whole prothrombin (Table 1). These results suggest that at least some of the influence of an acidic lipid membrane on the Pre2 domain of prothrombin arises from a membrane-induced change in the previously unrecognized interaction between the Pre2 and F1 domains.

Binding to a PS-Containing Membrane Couples Melting of a Portion of the F2 and F1 Domains. Thermal denaturation of the F1.2 domain of prothrombin is illustrated in Figure 4, with characteristic values recorded in Table 3. In Na_2EDTA-containing buffer, two distinct peaks are easily seen (Figure 4A): one with very low enthalpy at 45 °C corresponding to F2 melting (peak 1 in Table 3; see also Figure 5 and Table 2) and one at 60 °C corresponding to F1 melting [Figure 6 of Lentz *et al.* (1990); also summarized in Table 4). The transtions of the two domains of F1.2 are truly independent transitions as seen from the melting behavior of a mixture of F1 plus F2 in Na_2EDTA buffer (Figure 4B), which is nearly identical to that of F1.2. Addition of Ca^{2+} had no effect on the F2 portion of the F1.2 thermogram but shifted the F1 peak to 69 °C and increased its integrated enthalpy (Figure 4C), just as it did for F1 alone in the presence of Ca^{2+} (Lentz *et al.*, 1990; Table 4). Binding of F1.2 to 25% PS/POPC LUV in the presence of Ca^{2+} caused the loss of a fraction of the enthalpy from the F2 peak as well as the appearance of a very small shoulder just above the F1 peak (Figure 4E). In Table 3, this small shoulder is recorded as "peak 3". Although this shoulder was small, it was reproducibly observed in repeat scans of identically prepared samples containing different amounts of PS (10%, 15%, and 25% PS; Table 3). These results parallel the much larger effects seen with whole prothrombin (Lentz *et al.*, 1991; *cf.* Figure 2), suggesting that at least some of the influence of PS/POPC on prothrombin probably reflects an influence of the membrane on the stability of part or all of the F2 domain. This could reflect a direct interaction of F2 with a PS-containing membrane or a situation whereby F1 transmits the influence of the membrane to the F2 domain.

Fragment 2 Melting Is Significantly Altered in the Presence of Ca^{2+} and PS/POPC Membranes, Indicating a Direct F2 Domain/Membrane Interaction. The F1 domain of prothrombin is most often viewed as responsible for membrane binding *via* Ca^{2+}-mediated bridging of γ-carboxylated glutamic acid residues to acidic lipids (Dombrose *et al.*, 1979).

Table 1: Thermodynamic Characteristics of Bovine Prothrombin and Pre2 plus F1.2 Melting in the Presence and Absence of Ca^{2+} and Procoagulant Membranes

	deconvolution analysis												
		peak 1			peak 2			peak 3			peak 4		
protein	total ΔH^{cal}	T_m[a]	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R	T_m	ΔH^{cal}	R	T_m	ΔH^{cal}	R
II + EDTA	307				58.4	249	1.1	63.9	65	1.3			
II + Ca^{2+}	281				58.1	177	0.7	64.6	79	1.0	78.4	18	0.1
II and Ca^{2+} and PSPC (25%)	243				54.7	156	0.6	70.8	80	1.0	76.8	55	1.0
II and Ca^{2+} and PSPC (10%)	257				56.0	183	0.7	70.2	48	0.5	73.6	23	0.2
Pre2 + F1.2	212				53.3	143	0.7	59.8	76	1.2			
Pre2 + F1.2 and Ca^{2+}	241	47.1	19	0.2	52.4	204	1.1	68.7	56	0.4			
Pre2 + F1.2 and Ca^{2+} + PSPC and (25%)	213				49.6	139	0.9	70.7	51	0.3	75.4	26	0.2

[a] T_m is the midpoint of the transition in degrees celsius. [b] R is the ratio $\Delta H^{cal}/\Delta H^{vh}$, where ΔH^{cal} and ΔH^{vh} are the calorimetric and van't Hoff enthalpies in kilocalories per mole. Experimental error is estimated to be ±0.1 °C in T_m and ±15 kcal/mol in ΔH values.

Table 2: Thermodynamic Characteristics of Pre1 and Pre2 plus F1 in the Presence and Absence of Ca^{2+} and Procoagulant Membranes

	deconvolution analysis												
		peak 1			peak 2			peak 3			peak 4		
protein	total ΔH^{cal}	T_m[a]	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R	T_m	ΔH^{cal}	R	T_m	ΔH^{cal}	R
Pre1 + EDTA	197	54.0	33	0.3	58.6	158	0.6						
Pre1 + Ca^{2+}	229	53.4	28	0.3	57.8	200	0.8						
Pre1 + Ca^{2+} and PSPC (25%)	124	34.2	26	0.5	49.4	38	0.6				56.6	54	0.4
Pre2 + F2	192	46.6	37	0.3	51.7	159	1.0						
Pre2 + F2 and Ca^{2+}	210	(52.5	24	0.6)	51.2	164	1.0				71.8	24	0.1
Pre2 + F2 and Ca^{2+} and PSPC (25%)	210	30.4	121	1.4	47.4	73	0.5				69.0	25	0.2
Pre2 + F1	148				46.4	73	0.4	62.0	75	1.0			
Pre2 + F1 and Ca^{2+}	181				47.8	102	1.0	69.4	80	0.6			
Pre2 + F1 and Ca^{2+} and PSPC (25%)	170	42.3	30	0.3	47.4	27	0.2	67.5	64	0.8	70.9	49	0.5

[a] T_m is the midpoint of the transition in degrees celsius. [b] R is the ratio $\Delta H^{cal}/\Delta H^{vh}$, where ΔH^{cal} and ΔH^{vh} are the calorimetric and van't Hoff enthalpies in kilocalories per mole. Experimental error is estimated to be ±0.1 °C in T_m and ±15 kcal/mol in ΔH values.

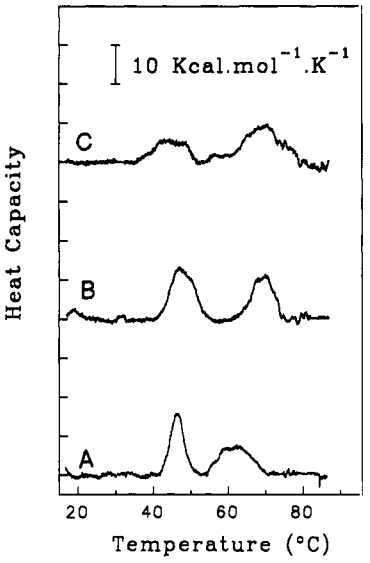

FIGURE 3: Thermal denaturation profiles of bovine prethrombin 2 plus fragment 1 in the absence and presence of Ca^{2+} and PS/POPC (25/75) membranes. (A) Pre2 (0.38 mg/mL) plus F1 (0.22 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (B) Pre2 (0.38 mg/mL) plus F1 (0.22 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (C) Pre2 (0.38 mg/mL) plus F1 (0.22 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$ and 10 mM PS/POPS (25/75) LUV.

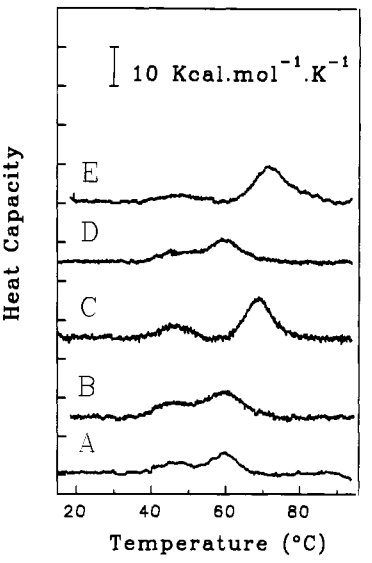

FIGURE 4: Thermal denaturation profiles of bovine fragment 1.2 in the absence and presence of Ca^{2+} and PS/POPC membranes. (A) F1.2 (0.6 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (B) F1 (0.22 mg/mL) and F2 (0.32 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (C) F1.2 (0.58 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (D) F1.2 (0.45 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA and 10 mM PS/POPC LUV. (E) F1.2 (0.58 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$ and 10 mM PS/POPC (25/75) LUV. The heating scan rate was 60 °C/h.

However, Bajaj *et al.* (1975) have reported weak binding of Ca^{2+} to the F2 domain of bovine prothrombin, making it necessary to consider also the direct effects of Ca^{2+} and membranes on this region. The results of such experiments (summarized in Figure 5 and Table 4) make it clear that Ca^{2+} had no effect on the denaturation of isolated F2. By contrast, the enthalpy of F2 melting increased 3–4-fold with essentially no change in T_m for a sample containing PS/POPC membranes with or without Ca^{2+} (Figure 5; Table 4). This demonstrates an unanticipated interaction between the F2 region of prothrombin and a PS-containing membrane in the presence or absence of Ca^{2+}. Thus, the effect of PS-containing membranes on F1.2 (see Figure 4E) could result in part from a direct interaction of F2 with the membrane.

Table 3: Thermodynamic Characteristics of F1.2 Melting in the Presence and Absence of Ca^{2+} and Procoagulant Membranes

		deconvolution analysis								
		peak 1			peak 2			peak 3		
protein	total ΔH^{cal}	T_m[a]	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R[b]
F1.2 + EDTA	105	45.5	30	0.4	59.4	62	0.8			
F1 + F2 + EDTA	116	45.3	36	0.4	59.7	82	1.1			
F1.2 + Ca^{2+}	139	45.5	36	0.5	68.9	95	0.9			
F1.2 and PSPC (25%)	88	46.6	23	0.3	59.6	66	1.0			
F1.2 and Ca^{2+} and PSPC (25%)	143	46.9	29	0.5	71.8	99	1.1	81.8	20	0.2
F1.2 and Ca^{2+} and PSPC (15%)	108	46.7	29	0.3	71.2	55	0.4	75.0	16	0.1
F1.2 and Ca^{2+} and PSPC (10%)	84	46.2	23	0.3	71.0	41	0.3	(53.6	21	0.3)

[a] T_m is the midpoint of the transition in degrees celsius. [b] R is the ratio $\Delta H^{cal}/\Delta H^{vh}$, where ΔH^{cal} and ΔH^{vh} are the calorimetric and van't Hoff enthalpies in kilocalories per mole. Experimental error is estimated to be ±0.1 °C in T_m and ±15 kcal/mol in ΔH values.

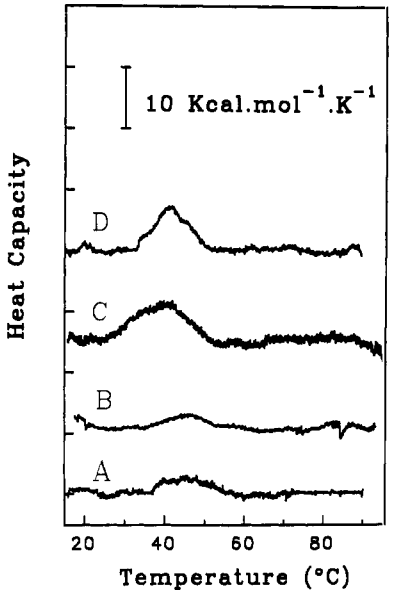

FIGURE 5: Thermal denaturation profiles of bovine fragment 2 in the absence and presence of Ca^{2+} and PS/POPC (25/75) membranes. (A) F2 (0.125 mg/mL) in MOPS buffer containing 0.1 mM Na_2-EDTA. (B) F2 (0.25 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (C) F2 (0.13 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA and 10 mM PS/POPC LUV. (D) F2 (0.14 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$ and 10 mM PS/POPC LUV.

Table 4: Thermodynamic Characteristics of F1 and F2 Melting in the Presence and Absence of Ca^{2+} and Procoagulant Membranes

		deconvolution analysis					
	total	peak 1			peak 2		
protein	ΔH^{cal}	T_m[a]	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R[b]
F1 + EDTA	55				59.0	61	0.9
F1 + Ca^{2+}	75				67.8	75	1.2
F1 and Ca^{2+} and PSPC (25%)	84				68.9	90	1.1
F1 and Ca^{2+} and PSPC (10%)	52				71.0	51	0.5
F2 + EDTA	24	45.8	24	0.6			
F2 + Ca^{2+}	21	45.5	22	0.9			
F2 + PSPC (25%)	96	41.2	70	1	(33.1	29	0.4)
F2 and Ca^{2+} and PSPC (25%)	78	43.1	42	0.6	(39.8	35	0.4)

[a] T_m is the midpoint of the transition in degrees celsius. [b] R is the ratio $\Delta H^{cal}/\Delta H^{vh}$, where ΔH^{cal} and ΔH^{vh} are the calorimetric and van't Hoff enthalpies in kilocalories per mole. Experimental error is estimated to be ±0.1 °C in T_m and ±15 kcal/mol in ΔH values.

The influence of Ca^{2+} and PS/POPC membranes on isolated F1 domain was reported previously (Lentz *et al.*, 1991) and is summarized in Table 4. While Ca^{2+} shifted the melting profile of F1 to about 68 °C and increased the enthalpy,

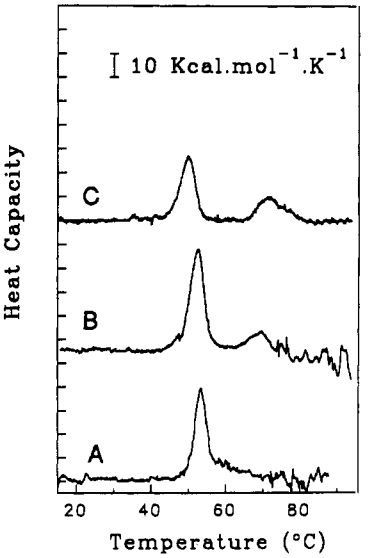

FIGURE 6: Thermal denaturation profiles of bovine prethrombin 2 plus fragment 1.2 in the absence and presence of Ca^{2+} and PS/POPC (25/75) membranes. (A) Pre2 (0.38 mg/mL) and F1.2 (0.35 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (B) Pre2 (0.38 mg/mL) and F1.2 (0.35 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (C) Pre2 (0.26 mg/mL) and F1.2 (0.24 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$ and 10 mM PS/POPC LUV. The heating scan rate was 60 °C/h.

addition of 25% PS/POPC membranes did little other than to increase the enthalpy of F1 melting. In the presence of membranes with lower surface concentrations of PS (10%), the enthalpy of F1 melting was lower, consistent with the proposal that prothrombin binding is mediated by binding of PS molecules to sites on F1 (Cutsforth *et al.*, 1989). If the endotherm associated with melting of F1 in the presence of Ca^{2+} and PS/POPC [not shown; see Lentz *et al.* (1991)] is subtracted from that for the melting of membrane-associated F1.2 (Figure 4E), a residual high-temperature peak remains (not shown). This demonstrates that this high-temperature component must be due to the effect of membranes on a portion of the F2 domain, not to the effect of membranes on F1.

Membrane Binding to F2 Alters Interaction with the Pre2 Domain. Several experiments were performed in order to test for the effects of Ca^{2+} or Ca^{2+} and PS/POPC membranes on domains of prothrombin other than the membrane binding F1 domain. These are illustrated in Figure 7 and summarized in Tables 2–4. The complete Prel fragment of prothrombin (Prel is prothrombin minus F1, *i.e.*, F2 covalently linked to Pre2) gave a denaturation endotherm with a main peak at 58 °C and a broad secondary peak at 54 °C. This contrasts somewhat with the denaturation of whole prothrombin, during which the Prel region melts at 58 °C in a simple two-state

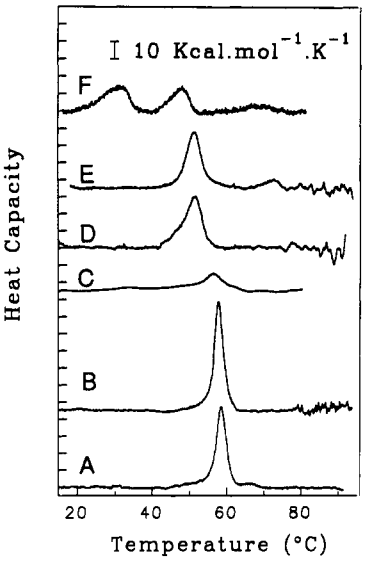

FIGURE 7: Thermal denaturation profiles of bovine prethrombin 1 in the absence and presence of Ca^{2+} and PS/POPC (25/75) membranes. (A) Pre1 (0.93 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (B) Pre1 (0.63 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (C) Pre1 (0.5 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$ and 10 mM PS/POPC LUV. (D) Pre2 (0.38 mg/mL) and F2 (0.13 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (E) Pre2 (0.38 mg/mL) and F2 (0.13 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (F) Pre2 (0.38 mg/mL) and F2 (0.13 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$ and 10 mM PS/POPC LUV.

fashion (R = 1.1; see Table 1). Apparently, the presence of the F1 domain encourages tight coupling between the F2 and Pre2 domains. Addition of Ca^{2+} to the Pre1 sample led to a slight increase in the enthalpy of melting but not to any significant change in the shapes or positions of the two endotherms. However, the presence of PS/POPC membranes produced dramatic effects on the denaturation profile of Pre1 (Figure 7C), resulting in a loss of enthalpy as well as very broad endotherms centered at 34 and 49 °C (Table 2). These two peaks may correspond to the melting of F2 in the presence of PS/POPC membranes and Ca^{2+} (Table 4). The loss of denaturation enthalpy, then, seems to reflect an altered structure of the Pre2 domain due to interaction of F2 with the PS/POPC membranes.

Putting Together the Pieces: Influence of Covalent Links between Domains. So far, we have identified an interaction between the F2 domain of prothrombin and a PS/POPC membrane as well as an interaction between the Pre2 and the F1 domains. The sum of these two effects could account roughly for the total high-temperature enthalpy change seen for the whole prothrombin bound to 25/75 PS/POPC membranes (Figure 2C). However, the effect of membranes on prothrombin is not exactly the sum of these two interactions. This is not a complete surprise in that the interaction of two isolated protein fragments does not require that they interact in the intact protein. A portion of a protein may have a different conformation when covalently attached to the rest of the protein than when proteolytically separated from its normal surroundings. To test for the importance of covalent attachment between domains, denaturation profiles were determined for equimolar mixtures of Pre2 plus F1.2. The results are summarized in Figure 6 and Table 1. The most striking feature of these thermograms is how similar they are in general appearance to those for whole prothrombin shown in Figure 2. Since Pre2 is so unstable as not to display a denaturation endotherm (Ploplis *et al.*, 1981; unpublished observations of S. Tendian and B. Lentz), the interactions between F2 and Pre2 (Myrmel *et al.*, 1976; Stevens *et al.*, 1993) are apparently essential for stabilizing the native structure of whole prothrombin.

Despite the similarity of this two-peptide system to whole prothrombin, there are detailed differences between the two that deserve mention. First, we note that all the domains of Pre2 and F1.2 melt with lower enthalpy in the presence of PS/POPC membranes than do those of whole prothrombin, although this difference is not clearly outside the experimental uncertainty in these numbers. Second, the main endotherm is shifted somewhat toward low temperatures and involves a bit less enthalpy (Table 1) than in whole prothrombin. Third, in the presence of Ca^{2+}, there appears a minor shoulder on the high-temperature edge of the main endotherm. Experiments presented in Figure 5 and summarized in Table 4 suggest that this endotherm derives from F2 denaturation. Fourth, Ca^{2+} shifts the F1 peak (peak 3 in Table 1) to a temperature similar to that observed in F1.2 or in F1 alone, but higher than that for whole prothrombin. Apparently, cutting the peptide bond between F2 and Pre2 decouples the F1 domain from the rest of the prothrombin molecule.

When the bond between the Pre2 and F2 domains is cut, there remains sufficient interaction between these domains to stabilize the Pre2 domain so that it melts in a two-state fashion at 51–52 °C (Figure 7D; Table 2). As mentioned previously, Pre2 itself is so unstable that no denaturation endotherm can be detected [*cf.* Ploplis *et al.* (1981)]. The denaturation profile of the mixed F2 and Pre2 domains is altered in the presence of Ca^{2+} (Table 2); a portion of the total denaturation enthalpy shifts to 72 °C while another portion remains associated with the Pre2 peak at 52 °C (Figure 7E; Table 2). Note that no Ca^{2+}-induced shift was detected in F2 alone or for whole Pre1 (Table 2). Apparently, both Pre2–F2 and Ca^{2+}–F2 interactions are required for the observed change in melting profile, but a strong, covalent coupling between F2 and Pre2 prevents it. At this point, it is not clear whether this result reflects a change in a portion of the F2 or the Pre2 domains. Another example of the importance to interdomain interactions in prothrombin of the covalent attachment of Pre2 and F2 is seen by comparing the effects of PS/POPC membranes on denaturation of Pre2 and F2 (Figure 7F; Table 2) with their effects on whole Pre1 (Figure 7C; Table 2). It appears that the F2–membrane interaction that we have demonstrated influences very differently the interactions of F2 with other domains as well as the stability of other domains when the constraints of the F2–Pre2 covalent linkage are relieved.

Finally, note the noise in the high-temperature region of scans A and B of Figure 6 and scans D and E of Figure 7. Noise like this was observed mainly in samples containing free Pre2 domain. Since such noise often accompanies aggregation at high temperature, this may reflect unfolding and aggregation of the relatively unstructured Pre2 domain. It may be that the peptide linkage between F1.2 and Pre2 prevents the unstable Pre2 domain from completely unfolding following denaturation of the F1 and F2 domains of whole prothrombin. It is of note that this high-temperature noise occurred only in samples free of membranes. It may be, then, that the portions of Pre2 and F2 domains affected by interaction with a membrane are the source of aggregation at high temperature.

Conclusions. The results summarized here demonstrate that thermal denaturation as monitored by DSC can provide considerable information about the effects of PS/POPC membranes on prothrombin structure and interdomain in-

teractions. Indeed, our results lead to at least five novel observations about the domain structure of bovine prothrombin and how this domain structure influences the interaction of this molecule with membranes and Ca^{2+}. First, F1 and F2 appear to be largely independent domains except for small effects on the thermal stability of F1 of an intact F2–Pre2 covalent bond and of an F2–membrane interaction. Second, F2 displays a Ca^{2+}-independent interaction with PS/POPC membranes. This may be responsible for the Ca^{2+}-independent membrane interaction of whole prothrombin that we have previously described as likely located in the Pre1 domain and probably the F2 domain (Tendian *et al.*, 1991, 1994). Third, we have located a previously undescribed interaction between the F1 and Pre2 domains that probably helps transmit the influence of PS/POPC membranes to the catalytic domain. These two observations provide a rational explanation for the previously described (Lentz *et al.*, 1990) specific effects of PS/POPC membranes on prothrombin. Fourth, our results make it clear that the prothrombin molecule has a highly cooperative structure, with PS/POPC-induced changes in membrane binding domains influencing the thermodynamic state of other regions of the molecule *via* a combination of covalent and noncovalent interdomain interactions. Finally, the thermal denaturation of the catalytic or Pre2 domain of prothrombin suggests that this region of prothrombin consists of at least three conformationally linked domains, two of which are distinguished by their abilities to bind to other domains (F1 and F2).

The observations enumerated above extend our previous effort (Lentz *et al.*, 1991) and support the conclusions and model presented at the beginning of this section. This model can be summarized as follows. Prothrombin binds Ca^{2+} in at least two of its three major proteolytically-generated domains: the F2 and F1 domains; the latter experiences a conformational change upon binding Ca^{2+}. The third proteolytically generated domain of prothrombin is the catalytic or Pre2 domain. This large and complex domain serves several functions and should, therefore, contain several functional subdomains. When Ca^{2+}-decorated prothrombin binds to PS/POPC membranes, conformational shifts occur in at least two subdomains of the Pre2 domain: one that is known to interact with the F2 domain and another that is shown here to interact with the F1 domain. Thus, the influence of specific membrane lipids is transmitted to the catalytic domain *via* both the F2 and F1 domains. While these interactions have been demonstrated with a variety of proteolytically generated fragments of prothrombin, the exact effect of membranes on the whole prothrombin molecule apparently requires the covalent linkage between F2 and Pre2. Thus, the previously examined kinetics of cleavage of the bond Arg^{323}–Ile^{324} by the membrane-assembled prothrombinase (Nesheim & Mann, 1983; Krishnaswamy *et al.*, 1987) may not be the same as the kinetics of cleavage of this bond in intact prothrombin.

Finally, we must note that the results that have led to this model are thermodynamic in nature and cannot demonstrate the conformational shifts postulated in the F2 and Pre2 domains. However, the observed membrane-induced shifts in denaturation temperature are consistent with shifts in the thermodynamic state (and, by inference, the conformational state) of these domains. The model is also consistent with our previous spectroscopic results demonstrating secondary structural changes induced in the Pre1 region of prothrombin on binding to PS-containing membranes (Wu & Lentz, 1991). This effect was shown to be PS-specific (Wu & Lentz, 1991; Lentz *et al.*, 1991) and, therefore, likely to involve sites on the protein specific for this lipid (Cutsforth *et al.*, 1989; Lentz *et al.*, 1991). We have shown elsewhere that PS-containing membranes produce membrane-bound prothrombinase complexes with greater intrinsic catalytic activity than other acidic lipid membranes (Pei *et al.*, 1993). We suggest that the origin of this acidic lipid specificity in the prothrombinase complex may reside in the conformational shifts and domain rearrangements indicated in the model shown in Figure 1.

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