Membrane binding induces lipid-specific changes in the denaturation profile of bovine prothrombin

A scanning calorimetry study

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ABSTRACT Prothrombin denaturation was examined in the presence of Na_2EDTA, 5mM $CaCl_2$, and $CaCl_2$ plus membranes containing 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) in combination with either bovine brain phosphatidylserine (PS) or 1,2-dioleoyl-phosphatidylglycerol (DOPG). Heating denaturation of prothrombin produced thermograms showing two peaks, a minor one at ~59°C previously reported to correspond to denaturation of the fragment 1 region (Ploplis, V. A., D. K. Strickland, and F. J. Castellino 1981. *Biochemistry.* 20:15–21), and a main one at ~57–58°C, reportedly due to denaturation of the rest of the molecule (prethrombin 1). The main peak was insensitive to the presence of 5mM Ca^{2+} whereas the minor peak was shifted to higher temperature (T_m ~65°C) by Ca^{2+}. Sufficient concentrations of POPC/bovPS (75/25) large unilamellar vesicles to guarantee binding of 95% of prothrombin resulted in an enthalpy loss in the main endotherm and a comparable enthalpy gain in the minor endotherm accompanying an upward shift in peak temperature (T_m ~73°C). Peak deconvolution analysis on the prothrombin denaturation profile and comparison with isolated prothrombin fragment 1 denaturation endotherms suggested that the change caused by POPC/PS vesicles reflected a shift of a portion of the enthalpy of the prethrombin 1 domain to higher temperature (T_m ~77°C). The enthalpy associated with this high-temperature endotherm increased in proportion to the surface concentration of PS. By contrast, POPC/DOPG (50/50) membranes shifted the prethrombin 1 peak by 4°C to a lower temperature and the fragment 1 peak by 5°C to a higher temperature. The data lead to a hypothesis that the fragment 1 and prethrombin 1 domains of prothrombin do not denature quite independently and that binding of prothrombin to acidic-lipid membranes disrupts the interaction between these domains. It is further hypothesized that PS containing membranes exert the additional specific effect of decoupling the denaturation of two subdomains of the prethrombin 1 domain of prothrombin.

INTRODUCTION

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 and 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 acidic lipid membranes. Intrinsic fluorescence quenching experiments (Nelsestuen, 1976; Prendergast and Mann, 1977), circular dichroism experiments (Bloom and Mann, 1978; Marsh et al., 1979), antibody binding experiments (Borowski et al., 1986), and differential scanning calorimetric (DSC)[1] studies (Ploplis et al., 1981) all have indicated that the NH_2-terminal third of prothrombin (termed fragment 1) undergoes one or two Ca^{2+}-induced conformational changes that are necessary for binding to acidic lipid membranes (Jackson et al., 1975; Nelsestuen, 1976). However, no data exist to determine whether the conformation or domain organization of prothrombin on a membrane is the same as in solution.

Ploplis et al. (1981) used differential scanning calorimetry to conclude that the fragment 1 and prethrombin 1 (prothrombin without fragment 1) regions of bovine prothrombin denatured roughly independently of each other, i.e., that they behave as independent domains. Addition of Ca^{2+} shifted the transition temperature of the fragment 1 domain but had little effect on the prethrombin 1 peak, consistent with the spectroscopic evidence mentioned above for a Ca^{2+}-induced conformational shift in the fragment 1 region of prothrombin. This result suggested to us that changes in domain organization and/or conformatin induced by binding to membranes might be revealed as shifts in the denaturation heat capacity profile of membrane-bound prothrombin relative to that for solution phase prothrombin. The results presented here indicate that some

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[1]*Abbreviations used in this paper:* DEAE-cellulose, diethylaminoethylcellulose; DOPG, 1,2-dioleoyl-3-*sn*-phosphatidylglycerol; DSC, differential scanning calorimetry; LUV, large, unilamellar vesicle(s); MOPS, 3-[*N*-Morpholino] propane-sulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetic acid; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; PS, bovine brain phosphatidylserine; *R*, ratio of the calorimetric to van't Hoff enthalpies; S-2238, phenylalanyl-pipecolyl-arginine-p-nitroaniline (synthetic substrate for thrombin).

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coupling does exist between the denaturation of the fragment 1 and prethrombin 1 domains of prothrombin and that this coupling is disrupted by binding to acidic-lipid membranes. Our results further show a specific effect of phosphatidylserine on the denaturation profile of the prethrombin 1 domain of prothrombin.

MATERIALS AND METHODS

Materials

Calcium chloride was reagent grade from Fisher Chemicals (Fair Lawn, NJ). Bovine brain phosphatidylserine (PS), 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) and 1,2-dioleoyl-3-*sn*-phosphatidylglycerol (DOPG) were purchased from Avanti Biochemicals (Birmingham, AL). The thrombin-specific substrate (phenylalanyl-pipecolyl-arginine-p-nitroaniline, S-2238) was purchased from Helena Laboratories (Beaumont, TX). All other chemicals were American Chemical Society (ACS) reagent grade or the best available grade; all solvents were HPLC grade.

Protein isolation, purification, and characterization

Bovine prothrombin was isolated by barium citrate precipitation from freshly collected bovine plasma (Mann, 1976; Tendian and Lentz, 1990). The barium citrate precipitate was a gift from Dr. Richard Hiskey of the Chemistry Department at the University of North Carolina at Chapel Hill (Chapel Hill, NC). Prothrombin obtained in this manner was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis and stained by Kodavue (Eastman Kodak, Rochester, NY) to reveal only one major band and no more than two very minor bands. A final purification step was performed one day before an experiment by HPLC on a Perkin-Elmer Isopure LC system using a Mono Q HR 5/5 ion exchange column (Pharmacia, Norwalk, CN). This step removed small quantities ($<10\%$) of proteolysis products that occasionally formed during storage (Tendian and Lentz, 1990).

Bovine prothrombin fragment 1 was prepared by thrombin proteolysis of prothrombin induced by *Echis Carinatus* venom (Sigma Chemical Co., St. Louis, MO), followed by chromatography on DEAE-cellulose and P-100 gel filtration columns as described elsewhere (Downing et al., 1975). Fragment 1 preparations were evaluated by sodium dodecylsulfate polyacrylamide gel electrophoresis to test for purity and by Ca^{2+}-induced fluorescence quenching (Nelsestuen, 1976; Prendergast and Mann, 1977) to test for a native conformation.

Prothrombin and prothrombin fragment 1 concentrations were determined from the absorbance at 280 nm after correcting for Rayleigh scattering using the absorbance at 320 nm. The extinction coefficients used were 1.44 (ml mg^{-1} cm^{-1}) for bovine prothrombin and 1.05 for bovine prothrombin fragment 1 (Mann, 1976).

Phospholipid vesicles

Two types of large unilamellar vesicle (LUV) preparations were used for these studies, with the results being the same for both. Reverse evaporation vesicles were prepared by the method of Szoka et al. (1980), whereas extruded LUV were prepared according to the procedure of Mayer et al. (1986). Some experiments were performed with small, unilamellar vesicles (Barenholz et al., 1977) so as to compare with the spectroscopic secondary structure determinations of Wu and Lentz (1991). In all cases, 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 benzene, the solution frozen, and lyophilized for at least eight hours 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 50/50 DOPG/POPC and 25/75 bov PS/POPC. Some experiments were performed with 10/90 or 17/83 bov PS/POPC to test for the effects of different surface concentrations of PS.

Differential Scanning Calorimetry

DSC measurements were performed on a MC-2 biological microcalorimeter (Microcal, Inc., Northampton, MA) equipped with a Keithly 150B amplifier (Keithley Instruments, Inc., Cleveland, OH). Samples were deaerated a half-hour before being loaded into the calorimeter cell with a Hamilton microliter/gastight syringe (Hamilton Company, Reno, NV). The buffer for calorimetry scans was 50 mM 3-[*N*-Morpholino] propane-sulfonic acid (MOPS; Sigma Chemical Co., St. Louis, MO), 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 before 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 the thermograms was detected at or below this scan rate. 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. Calorimetric data were converted to heat capacity (kcal/mol/°K) versus temperature profiles and endothermic heat capacity peaks were integrated using the continuous baseline algorithm (Sturtevant, 1987). Multiple peaks were resolved using software provided by Microcal, Inc. (Northampton, MA). All experiments were performed at least in duplicate and in some cases up to five times for separate samples to be certain that subtle effects were reproducible. Data are presented for representative experiments.

RESULTS

Prothrombin denaturation profiles.

When heated in the buffer system used here, bovine prothrombin denatured in two discernable steps to yield a heat capacity profile with a sharp endothermic peak and an associated high temperature endothermic shoulder (Fig. 1 *A*). As a control, denaturation scans were performed on pure prothrombin at several different scan rates (20, 40, 60°C/h) with the result that neither the shape nor the position of the main endotherm was altered significantly (e.g., <0.4°C shift in T_m). The deconvolution analysis of Table 1 documents the resolved individual peak temperatures and enthalpies as estimated by the deconvolution algorithms provided by Microcal. These deconvolution algorithms resolve overlapping peaks according to three basic models: (*a*)

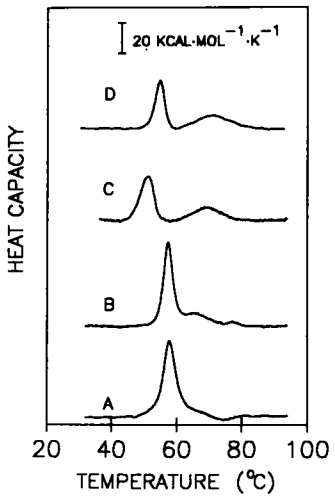

FIGURE 1 Thermal denaturation profiles of bovine prothrombin in the presence and absence of Ca^{2+} and different acidic lipid membranes. (*A*) Prothrombin (0.98 mg/mL) in the MOPS buffer containing 5 mM Na_2EDTA. (*B*) Prothrombin (0.69 mg/mL) in MOPS buffer containing 5 mM $CaCl_2$. (*C*) Prothrombin (0.62 mg/mL) and 3 mM DOPG/POPC LUV in MOPS buffer containing 5 mM $CaCl_2$. (*D*) Prothrombin (0.85 mg/mL) and 10 mM bovine PS/POPC LUV in MOPS buffer containing 5 mM $CaCl_2$. In all cases, the heating scan rate was 60°C/h.

independent, two-state transitions, (*b*) independent, non-two-state transitions, and (*c*) linked, or sequential, two-state transitions according to the model described by Friere and Biltonen (1978). The least restrictive model (independent, non-two-state transitions) was generally used. In this case, the ratio of the integrated calorimetric enthalpy to the van't Hoff enthalpy (R) for any resolved peak provides information about the nature of the denaturation process associated with that peak (Strurtevant, 1987). A ratio close to one indicates a simple, two-state, all-or-nothing denaturation event characteristic of a simple globular protein (Sturtevant, 1987). This behavior is indicated for the main endotherm of prothrombin denaturation (peak 1, Table 1). A ratio greater than one, as seen for the minor peak (peak 2) of the prothrombin denaturation profile (Table 1), indicates that more than one two state transition is associated with the resolved endotherm.

When 5 mM Ca^{2+} was included in the sample buffer, Peak 1 was unaltered (Fig. 1 *B*, Table 1). The minor endotherm (peak 2) was shifted slightly to higher temperature, so that it became more clearly resolved from peak 1. The denaturation enthalpy of the minor endotherm decreased slightly in the presence of Ca^{2+} (Table 1). Ploplis et al. (1981) compared the denaturation profiles of whole prothrombin with the profiles of its proteolytic fragments to show that, to a first approximation, peak 1 was due to denaturation of the prethrombin 1 domain (COOH-terminal two thirds) of prothrombin, whereas peak 2 was associated with melting of the fragment 1 domain (NH_2-terminal one third containing the γ-carboxyglutamic acid residues thought to be responsible for membrane binding, see Fig. 2). Whereas our results are reasonably consistent with those of Ploplis et al., (1981) there are differences. First, our denaturation profiles

TABLE 1 Thermodynamic characteristics of the melting profiles of prothrombin and fragment 1

	Deconvolution Analysis							
	Total	Peak 1			Peak 2			
Protein	ΔH^{cal}*	T_m‡	ΔH^{cal}	R§	T_m	ΔH^{cal}	R	χ^2‖
Prothrombin + EDTA	320	57.8	184	1.0	59.7	142	2.2	1.4
Prothrombin + 5 mM Ca^{2+}	310	57.4	186	0.8	64.7	125	2.2	2.5
Prothrombin + Ca^{2+} and POPC	301	56.4	161	0.9	63.6	120	2.0	2.2
Prothrombin + Ca^{2+} and PG/PC (50/50)	224	52.3	166	1.1	69.1	83	0.9	1.4
Prothrombin + Ca^{2+} and PS/PC (25/75)	243	54.8	114	0.6	72.6	129	1.8	2.5
Prothrombin + Ca^{2+} and PS/PC (17/83)	230	55.7	126	0.5	71.5	103	1.4	2.0
Prothrombin + Ca^{2+} and PS/PC (10/90)	257	56.1	183	0.7	71.7	74	0.8	2.0
Prothrombin F.1	55	—	—	—	59.0	61	0.9	1.4
F.1 + 5 mM Ca^{2+}	75	—	—	—	67.8	75	1.2	1.4
F.1 + Ca^{2+} and PG/PC	80	—	—	—	69.9	88	0.8	1.4
F.1 + Ca^{2+} and PS/PC	84	—	—	—	68.9	90	1.1	4.8

*, ΔH^{cal} and ΔH^{vh} are the calorimetric and van't Hoff enthalpies in kcal/mol; ‡, T_m is the midpoint of the transition in degree Celsius; §, R is the ratio, $\Delta H^{cal}/\Delta H^{vh}$; ‖, χ^2 calculated as $s^2/(1/2^*$ mean peak-to-peak noise$)^2$; Experimental error is estimated to be ±0.1°C in T_m and ± 15 kcal/mol in ΔH values.

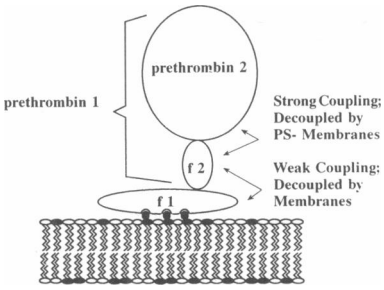

FIGURE 2 Illustration of the peptide fragment structure (and hypothesized domain structure) of bovine prothrombin, the largest fragment being *prethrombin 2,* which in combination with *fragment 2* (f2) is referred to as *prethrombin 1.* The NH_2-terminal 30% of the molecule containing Ca^{2+}-binding and membrane-binding sites is referred to as *fragment 1* (f1). Our hypothesis (see Discussion) for the effect of acidic-lipid membranes on prothrombin domain structure is also illustrated.

show peaks a few degrees lower than the profiles reported by these authors. This probably reflects the different ionic conditions and buffers used by us and by Ploplis et al. (1981). Second, as will be described in more detail below, our results cannot support the conclusion that the fragment 1 and prethrombin 1 domains denature completely independently.

Denaturation of membrane-bound prothrombin

To assess the effect of acidic lipid membranes on the denaturation behavior of prothrombin, appropriate membranes were included in samples at sufficient concentrations so that $\geq 95\%$ of prothrombin was bound, as judged from published binding constants (Nelsestuen and Broderius, 1977). When DOPG/POPC (50/50) LUV were included, peak 2 decreased in enthalpy and shifted to slightly higher temperature, whereas peak 1 broadened, decreased slightly in enthalpy, and shifted to lower temperature (Fig. 1 *C* and Table 1). Of particular note is the fact that the *R* value associated with peak 2 decreased from roughly two for soluble forms of prothrombin to one for the DOPG/POPC-bound form. One possible explanation for this could be a change in prothrombin interdomain interactions associated with binding to DOPG/POPC membranes.

Vesicles composed of bovine PS/POPC (25/75) had a different effect on the prothrombin denaturation profile, as seen in Fig. 1 *D*. The enthalpy of the peak 1 endotherm decreased somewhat more than it did upon addition of DOPG/POPC membranes. Of special note, however, was the fact that peak 2 was significantly broader and larger than observed with DOPG/POPC membranes (compare Fig. 1 *C* and *D*). As for prothrombin in the presence of 5 mM $CaCl_2$, this endotherm, when fit as a single transition, gave an *R* value close to two (Table 1). For this reason, we attempted a two-component fit of peak 2 and found it to be resolvable into two independent, two-state transitions (Fig. 3 and Table 2), one of which had roughly the characteristics of the peak 2 endotherm in the presence of DOPG/POPC membranes and one of which occurred at even higher temperature (see Table 2). A linked-domain, or sequential melting model (Freire and Biltonen, 1978) produced a significantly poorer fit to the PS/POPC data. When a similar independent, two-component analysis was performed on peak 2 data obtained from prothrombin bound to DOPG/POPC membranes, only a single endotherm, identical to the denaturation endotherm of DOPG/POPC bound fragment 1, was resolved (Table 2).

To explore further the origin and nature of the complex high temperature endotherm observed in the presence of PS/POPC membranes, we examined the denaturation of prothrombin bound to membranes containing decreasing surface concentrations of PS. Using previously determined binding constants (Nelsestuen and Broderius, 1977; Cutsforth et al., 1989), vesicle concentrations were adjusted to assure that $>95\%$ of prothrombin was membrane-bound in all cases. The resulting melting profiles are shown in Fig. 4. We analyzed the peak 2 endotherm of these melting profiles and found that the enthalpy of this peak increased with

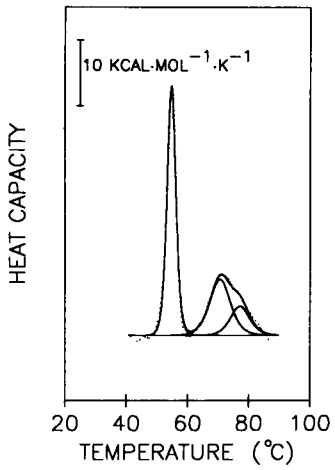

FIGURE 3 Thermal denaturation profiles of bovine prothrombin in the presence of 5 mM Ca^{2+} and 10 mM bovine PS/POPC LUV (data from Fig. 1 *D*) deconvoluted according to the independent, two-state model (see text). The experimental results can be identified by the noise in the data, whereas calculated endotherms are shown by smooth lines.

TABLE 2 **Two-component analysis of the peak 2 endotherm for prothrombin in the presence of Ca^{2+} and acidic lipid membranes**

	T_m	ΔH	R	T_m	ΔH	R
	°C	*kcal/mol*		°C	*kcal/mol*	
Two-component deconvolution analysis						
Prothrombin and Ca^{2+} + PS/POPC (25% mol)	70.8	80	1.0	76.8	55	1.0
Prothrombin and Ca^{2+} + PG/POPC (50% mol)	69.1	83	0.9	—	0*	—
Subtraction of membrane bound fragment 1‡						
Prothrombin and Ca^{2+} and PS/POPC				77.1	45	—
Prothrombin and Ca^{2+} and PG/POPC				—	0*	—

*No second endotherm was detected; ‡The denaturation profile of F. 1 and Ca^{2+} & PS/POPC (or PG/POPC) was subtracted from the peak 2 endotherm of PS/POPC (or PG/POPC)-membrane bound prothrombin (Fig. 7 in text).

membrane PS-content (Table 1 and dotted line in insert of Fig. 4). The shape of the peak 2 endotherm also changed with PS content, with the R value increasing from roughly one to nearly two as PS content increased. For this reason, we examined the ability of a two-component analysis to describe the peak 2 endotherm of each profile. Except at the lowest PS surface concentration examined (10 mol%), there was clear evidence of two components in the peak 2 endotherms, with the enthalpy of the high-temperature component of this pair

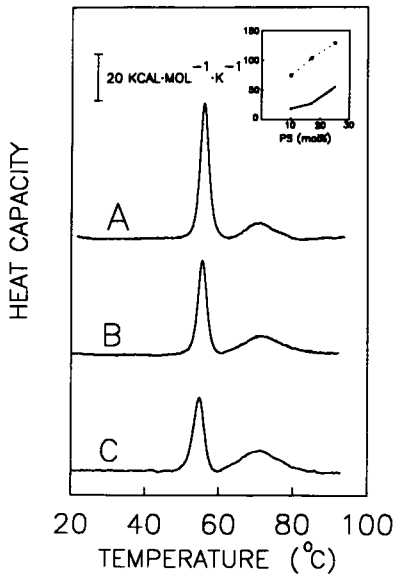

FIGURE 4 Thermal denaturation profiles of prothrombin in the presence of 5 mM $CaCl_2$ and bovPS/POPC LUV at different surface concentrations of PS. (*A*) 10 mol% PS (1.323 mg protein); (*B*) 17 mol% PS (1.466 mg protein); (*C*) 25 mol% PS (1 mg protein). Insert: PS dependence of the total peak 2 enthalpy (*dotted line*) and the enthalpy (*solid line*) associated with the high-temperature resolved peak (see Fig. 3) associated with the denaturation profiles shown in curves A, B, and C (units on the ordinate are kcal/mol).

observed to increase with increasing PS membrane contents (solid line in insert of Fig. 4). Even for the 10 mol% sample, careful examination of the peak 2 endotherm revealed a slight asymmetry indicative of two overlapping peaks (see Fig. 4*A*). The lower temperature component always approximated the behavior of the peak 2 endotherm for prothrombin bound to DOPG/POPC membranes (Fig. 1 *C*). In summary, then, the denaturation profile for prothrombin bound to membranes of low PS content approached the denaturation profile for prothrombin bound to DOPG/POPC membranes. Increasing PS content broadened the peak 2 endotherm of bound prothrombin at the same time that it sharpened slightly the peak 1 endotherm (see R values in Table 1).

As further controls to test whether irreversible aggregation might account for the peak 2 endotherm observed in the presence of PS/POPC membranes, denaturation experiments were carried out in the presence of PS/POPC membranes with or without Ca^{2+} at different scan rates (20, 45, and 60°C/h) and at different lipid (2 and 10 mM) and protein concentrations (0.3–1 mg/ml). As is illustrated for the two scans at different scan rate shown in Fig. 5, neither the shape nor the peak position of either the peak 1 or peak 2 endotherms were altered significantly by these parameters. Denaturation profiles performed in the presence of PS/POPC membranes but in the absence of Ca^{2+} were identical to profiles of prothrombin alone. Finally, activity measurements carried out under conditions that mimic a 30°C/h calorimetric experiment showed that prothrombin remained fully activatible to thrombin up to temperatures just above the peak 2 T_m but then lost activatibility only slowly so that significant activity (>50% for prothrombin in the presence of Ca^{2+}, 30% in the presence of POPC membranes, and 20% in the presence of PS/POPC membranes) remained even at 80°C (Pei, G., and B. R. Lentz, unpublished observations).

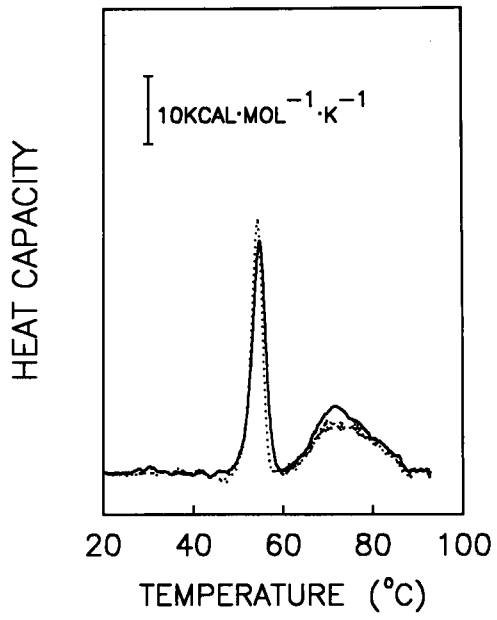

FIGURE 5 Thermal denaturation profiles of bovine *prothrombin and* 10 mM bovine PS/POPC LUV in MOPS buffer containing 5 mM $CaCl_2$ with 0.86 mg/ml protein at a scan rate of 60°C/h (*solid line*) and 1.02 mg/ml protein at a scan rate of 20°C/h (*dotted line*).

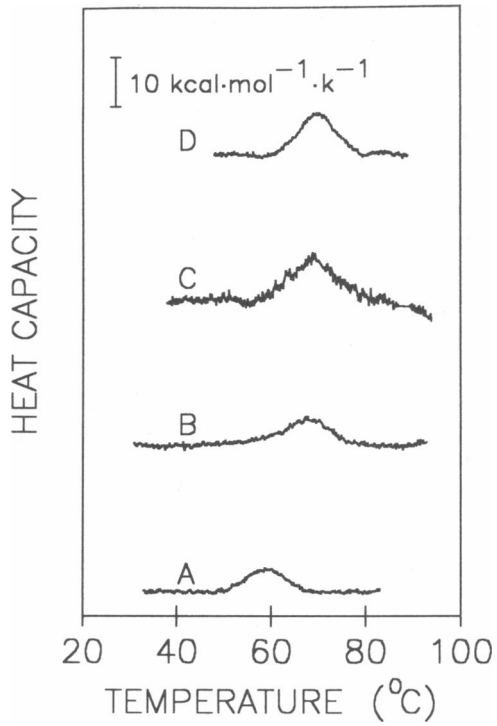

FIGURE 6 Thermal denaturation profiles of bovine prothrombin fragment 1 in the presence and absence of Ca^{2+} and different acidic lipid membranes. (*A*) Fragment 1 (0.45 mg/ml) in the MOPS buffer containing 5 mM Na_2EDTA. (*B*) Fragment 1 (0.24 mg/ml) in MOPS buffer containing 5 mM $CaCl_2$. (*C*) Fragment 1 (0.18 mg/ml) and 3 mM DOPG/POPC LUV in MOPS buffer containing 5 mM $CaCl_2$. (*D*) Fragment 1 (0.21 mg/ml) and 10 mM bovine PS/POPC LUV in MOPS buffer containing 5 mM $CaCl_2$. In all cases, the heating scan rate was 60°C/h.

Fragment 1 denaturation profiles

To better interpret the experiments with whole prothrombin, analogous experiments were performed with the isolated fragment 1 domain of prothrombin. The results of these are summarized in Fig. 6 and Table 1. Several aspects of these results bear examination. First, the unfolding of isolated fragment 1 appears to be adequately described by a simple two-state model ($R \sim 1$). Except for DOPG/POPC-bound prothrombin, the peak 2 endotherm was never equivalent to the melting endotherm of isolated fragment 1 (see Table 1). This suggests that the peak 2 endotherm of whole prothrombin does not correspond rigorously to the melting of fragment 1 as an independent subdomain, as suggested by Ploplis et al. (1981). Instead, it appears that fragment 1 melting in whole prothrombin (except when bound to DOPG/POPC) is weakly coupled to the melting of another subdomain, probably fragment 2. Second, whereas Ca^{2+} altered the denaturation profile of fragment 1 significantly (Fig. 6 *B*) as previously reported (Ploplis et al., 1981), fragment 1 melting was less affected by membrane binding and was essentially independent of the membrane to which this peptide was bound (see Fig. 6, curves B, C, and D).

A third feature of our fragment 1 results bearing mentioning is the fact that they differ somewhat from those previously reported by Ploplis et al. (1981). These authors found a significantly larger enthalpy for fragment 1 denaturation than we have found. In addition, Ploplis et al. (1981) found that this enthalpy decreased with Ca^{2+} binding, whereas our results indicate a slight increase (Table 1). We can find no obvious explanation for this disagreement. All of our denaturation profiles were reproducible with different protein preparations and on two different microcalorimeters (the Microcal MC-2 and an older Tronac 710; Tronac Inc., Orem, UT). However, the methods of fragment 1 preparation used by Ploplis et al. (1981) and by us were slightly different. In addition, Ploplis et al. (1981) did not subject their preparation to the test of Ca^{2+}-induced fluorescence quench (Prendergast and Mann, 1977), as we did. We have observed anomalous membrane interactions with certain preparations of apparently chromatographically pure fragment 1 that did not fully reproduce this fluorescence quench. It might be, then, that a subtle

difference in fragment 1 preparations could explain the difference between our fragment 1 results and those of Ploplis et al. (1981).

Finally, except for DOPG/POPC membranes, the fragment 1 denaturation profile was not equivalent to peak 2 in the denaturation profile of whole prothrombin (see Table 1). The integrated area of this peak in the whole prothrombin profile was greater than that for the isolated fragment 1. In addition, except for the DOPG/POPC-bound prothrombin, the R value for peak 2 of prothrombin denaturation was close to two but nearly one for isolated fragment 1. This means that some portion of the prethrombin 1 domain denatures at a temperature close to that of fragment 1, except when prothrombin is bound to a DOPG/POPC membrane. It may be that interactions exist between the fragment 1 and prethrombin 1 domains of the prothrombin molecule and that binding to DOPG/POPC membranes alters these interdomain interactions and allows the fragment 1 domain to denature completely independently of its prethrombin 1 neighbor ($R \sim 1$). Ploplis et al. (1981) noted in their Discussion that certain aspects of their data also suggested such an interaction, although they preferred the first order approximation that these domains were independent.

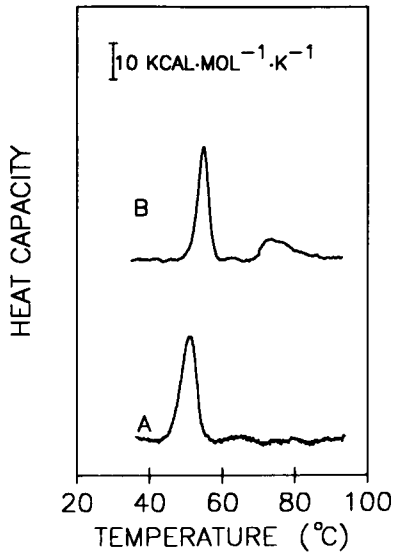

FIGURE 7 Thermal denaturation profiles of bovine prothrombin obtained from data in Fig. 1, *C* and *D*, after subtraction of the thermal denaturation endotherms of prothrombin fragment 1 (Fig. 6, *C* and *D*, respectively): (*A*) prothrombin in the presence of 5 mM Ca^{2+} and 3 mM DOPG/POPC LUV; (*B*) prothrombin in the presence of 5 mM Ca^{2+} and 10 mM bovine PS/POPC LUV.

PS-Specific denaturation changes

Analysis of our prothrombin denaturation profiles (see above) has suggested that binding to PS-containing membranes specifically shifted the denaturation of some portion of the prethrombin 1 domain to a higher temperature, leading to an apparent enhancement of the peak 2 endotherm. To test further this interpretation, the denaturation profile of membrane-bound fragment 1 was subtracted from the profile for membrane-bound, whole prothrombin, as shown in Fig. 7. The disappearance of the peak 2 endotherm for DOPG/POPC membranes (Fig. 7 *A*) shows that this endotherm in the prothrombin profile corresponds to melting of the membrane-bound fragment 1 domain which melts independently of the prethrombin 1 domain. The results for prothrombin bound to PS/POPC membranes were strikingly different (Fig. 7 *B*). Here, subtraction of the membrane-bound fragment 1 profile uncovered an apparent, high-temperature isotherm whose thermal characteristics were approximately those of the peak revealed by the two-component deconvolution procedure applied to peak 2 (Table 2). The slight high-temperature asymmetry in the high-temperature difference peak in Fig. 7 *B* and the small dip in the baseline on the low-temperature side of this peak probably reflect the limits of experimental reproducibility but might reflect a weak interaction between the domains accounting for peak 2 in PS/POPC-bound prothrombin. However, these results do confirm our two-component analysis (Table 2) and support the existence of a specific alteration in the subdomain organization of prethrombin 1 and/or in interdomain interactions induced by binding of prothrombin to PS-containing membranes.

DISCUSSION

Interpretation of denaturation endotherms

This paper reports a change in the denaturation profile of the prethrombin 1 region of prothrombin upon binding to acidic lipid-containing membranes. In addition, the results demonstrate a different effect on prothrombin denaturation of binding to PS/POPC as compared to DOPG/POPC membranes. It has long been recognized that, subject to certain assumptions, the denaturation profile of proteins contains information about the thermodynamic state of the protein at temperatures below the denaturation temperature (Privalov, 1979, 1982). Among the most troublesome of these assumptions is that of reversibility. As for most thermally induced protein denaturation, prothrombin sam-

ples that had been scanned to 90°C, cooled and scanned again, produced thermograms with greatly reduced denaturation endotherms, indicating that thermal unfolding was itself, or was accompanied by, a substantially irreversible process. Arguments have been put forward as to why the laws of reversible thermodynamics should apply to such irreversible processes (Privalov, 1982). The most convincing argument is simply that denaturing protein systems *behave* according to the laws of thermodynamics (Sturtevant, 1987), suggesting that thermal denaturation might follow a locally reversible pathway followed by an irreversible step. Freire et al. (1990) have analyzed the denaturation process in terms of a model that assumes a rapid reversible unfolding reaction followed by irreversible aggregation. These authors showed that the melting profile could be used to obtain thermodynamic information if the irreversible process was sufficiently slow that a significant fraction of the reversibly unfolded state existed above the unfolding transition. Friere et al. (1990) presented a kinetic analysis of their model to show that the diagnostic of an acceptably slow irreversible step was a transition endotherm whose peak temperature and shape were only slightly altered by increasing scan rate. Prothrombin denaturation scans, in the presence or in the absence of PS/POPC or POPC membranes (with or without Ca^{2+}) showed little or no variation with scan rate (see Results and Fig. 5). Other unpublished results (Pei, G., and B. R. Lentz) show that a large fraction of prothrombin molecules retains the ability to be activated to thrombin well above the melting endotherm. These observations both indicate that our denaturation profiles, even in the presence of membranes, should be interpretable in thermodynamic terms. In what follows, we take this position.

Effect of membrane and Ca^{2+} binding on prothrombin fragment 1

With due regard for the caveats raised above, we can now discuss the implications of our results in terms of the influence of Ca^{2+} and membrane binding on the thermodynamic state of prothrombin in solution.

Our results demonstrate a Ca^{2+}-induced shift in the T_m of fragment 1 denaturation (Table 1). Assuming that Ca^{2+} does not alter the stability of the denatured state, the explanation must be that binding somehow stabilizes the undenatured state. This stabilization might come about by stabilizing the native structure, by inducing a new, more stable structure, or, as pointed out by Fukada et al. (1983) by favoring protein multimer formation. Whereas there is some support for the Ca^{2+}-induced dimerization of fragment 1 or prothrombin (Jackson et al., 1987), reported dimerization constants would make it minimal under our conditions. There is, however, overwhelming direct structural evidence for a Ca^{2+}-induced shift in fragment 1 structure (Nelsestuen, 1976; Bloom and Mann, 1978; Österberg et al., 1980; Borowski et al., 1986; Soriano-Garcia et al., 1989), making this the interpretation that we favor.

Membrane binding of the isolated fragment 1 portion of prothrombin increased slightly the enthalpy and the peak temperature associated with the denaturation profile of this peptide (Fig. 6 and Table 1). In analogy to an earlier study of the effects of ligand binding on denaturation (Fukada et al., 1983), a reasonable interpretation would be that binding to an acidic lipid membrane had only a very small influence on the thermodynamic state of the fragment 1 portion of prothrombin. Our data suggest an enthalpy of fragment 1 binding to acidic lipid membranes of roughly 13–15 kcal mol^{-1-} (compare denaturation enthalpies for fragment 1 and Ca^{2+} and for fragment 1 and Ca^{2+} and membranes in Table 1). Recent Fourier transform infrared spectroscopic studies in our laboratory (Wu and Lentz, 1991) also failed to detect secondary structural changes in prothrombin fragment 1 on binding to PS or PG containing membranes.

Hypothesis for the effect of acidic lipid membranes on prothrombin domains

It is not possible to interpret unambiguously the effect of membrane binding on the denaturation of the whole prothrombin molecule at this stage. It is evident from our results, however, that prothrombin does not denature in two, independent, two-state transitions. Even in the absence of membranes, the peak 2 endotherm reflected the denaturation of more than an independent fragment 1 domain (see R values and compare isolated fragment 1 endotherm to peak 2 endotherm in Table 1). Although an earlier study (Ploplis et al., 1981) concluded that fragment 1 and prethrombin 1 denatured as two independent domains, these authors noted that "interaction between the two domain regions may, in fact, occur." In addition, Ploplis et al. (1981) suggested a tight coupling between the denaturation of fragment 2 and prethrombin 2 (prethrombin 1 *minus* fragment 2). An hypothesis to rationalize our data and the observations of Ploplis et al. (1981) can be formulated in terms of the thermodynamic analysis of denaturation of multidomain proteins proposed by Brandts et al. (1989): (*a*) fragment 2 and prethrombin 2 denaturation are tightly coupled by strong interactions between these domains (Myrmel et al., 1976); (*b*) fragment 1 and fragment 2 domains are also coupled in their denaturation behavior; (*c*) binding of fragment 1 to acidic lipid membranes weakens or eliminates this fragment 1–fragment 2 coupling; (*d*) specific binding of PS to a site on fragment 2

raises the melting temperature of fragment 2 and alters the interaction between prethrombin 2 and fragment 2. This hypothesis is summarized and illustrated in Fig. 2. Although our data are insufficient to prove this model at this point, there are precedents for many of its features. For instance, the 77°C endotherm accounted for ~23% of the total enthalpy of prethrombin 1 denaturation. Because the molecular weight for prethrombin 1 is about 50,000 D, a domain of roughly 11,000 D (~100 residues) could account for the high temperature endotherm. Fragment 2, the first 118 residues of prethrombin 1 (Mann et al., 1981), would be a good candidate. Other results from this laboratory suggest the existence of a PS-specific, Ca^{2+}-independent interaction of the prethrombin 1 portion of prothrombin (possibly the fragment 2 region) with acidic lipid membranes (Tendian and Lentz, 1990). The lower T_m of peak 1 in the presence of both PS and PG containing membranes is seen as reflecting the partial uncoupling of fragment 1 and prethrombin 1 domains. The increase in the cooperative nature of the peak 1 endotherm as well as the slight decrease in the T_m of this endotherm at increased PS content can be argued to be consistent with PS-mediated stabilization of the fragment 2 subdomain which alters the interaction between the prethrombin 2 and fragment 2 subdomains. A more extensive analysis of the effects of membrane binding on the domain structure of prothrombin and its fragments will be necessary in order to carefully test this hypothesis, especially the possibility of fragment 1–framgent 2 coupling.

Lipid specificity and implications for the lipid specificity of prothrombin activation

The second major observation stemming from this study is the demonstration that PS/POPC and DOPG/POPC membranes had different effects on the denaturation profile of prothrombin. The most dramatic evidence of lipid specificity comes from the increase in high-temperature endotherm in proportion to the surface concentration of PS (Fig. 4), whereas binding to DOPG/POPC membranes induced no such high-temperature endotherm even at a DOPG surface concentration of 50 mol% (Fig. 1 and Table 1). Although the thermodynamic changes demonstrated here can not be used to support PS-specific structural changes, this thermodynamic analysis is consistent with analysis of Fourier transform infrared spectra showing a small increase in the proportion of ordered secondary structure of bovine prothrombin bound to PS containing but not DOPG containing membranes (Wu and Lentz, 1991). A similar PS specificity is exhibited by the enzymatic properties of the membrane-assembled human prothrombinase (Pei, G., D. Powers, and B. Lentz, manuscript in preparation). Despite the species difference, this correlation suggests that specific-lipid-induced conformational shifts in prothrombin associated with membrane binding could have functional significance. Demonstrating this membrane-dependent structure-function coupling will require further study, including a more extensive prothrombin domain analysis.

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REFERENCES

Barenholz, Y., D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson, and F. D. Carlson. 1977. A simple method for the preparation of homogeneous phospholipid. *Biochemistry.* 29:2806–2810.

Bloom, J. W., and K. G. Mann. 1978. Metal ion induced conformational transitions of prothrombin and prothrombin fragment 1. *Biochemistry.* 17:4430–4438.

Borowski, M., B. C. Furie, S. Bauminger, and B. Furie. 1986. Prothrombin requires two sequential metal-dependent conformational transitions to bind phospholipid. *J. Biol. Chem.* 261;14969–14975.

Brandts, J. F., C. Q. Hu, L.-N. Lin, and M. T. Mas. 1989. A simple model for protein with interacting domain: applications to scanning calorimetry data. *Biochemistry.* 28:8588–8596.

Chen, P. S., T. Y. Toribara, and H. Warner, 1956. Microdetermination of Phosphorus. *Anal. Chem.* 28:1756–1758.

Cutsforth, G. A., R. N. Whitaker, J. Hermans, and B. R. Lentz. 1989. A new model to describe extrinsic protein binding to phospholipid membranes of varying composition: application to human coagulation proteins. *Biochemistry.* 28:7453–7459.

Downing, M. R., R. J. Butkowski, M. M. Clarke, and K. G. Mann, 1975. Human prothrombin activation. *J. Biol. Chem.* 250:8897–8906.

Freire, E., and R. L. Biltonen. 1978. Statistical mechanical deconvolution of thermal transitions in macromolecules. I. Theory and applications to homogeneous systems. *Biopolymers.* 17:463–479.

Freire, E., W. W. van Osdol, O. L. Mayorga, and J. M. Sanchez-Ruiz. 1990. Calorimetrically determined dynamics of complex unfolding transitions in proteins. *Annu. Rev. Biophys. Chem.* 19:159–188.

Fukada, H., J. M. Sturtevant, and F. A. Quiocho, 1983. Thermodynamics of binding of L-Arabrinose and of D-Galactose to the L-Arabrinose-binding protein of *Escherichia coli. J. Biol. Chem.* 258:13193–13198.

Jackson, C. M., C. T. Esmon, S. N. Gitel, W. G. Owen, and R. A. Henrikson. 1975. The conversion of prothrombin to thrombin: the function of the propiece of prothrombin. *In* Prothrombin and

Related Coagulation Factors. H. C. Hemker, and I. J. Voltkamp, editors. Lieden University Press, Leiden, The Netherlands. 59–88.

Jackson, C. M., G. M. Brenckle, P. J. Hogg, and D. J. Winzor. 1987. Evidence for self-association of prothrombin fragment 1 in the absence of calcium ions. *J. Biol. Chem.* 262:13472–13475.

Mann, K. G. 1976. Blood clotting enzymes [13]: Prothrombin *Methods Enzymol.* 45:123–156.

Mann, K. G. 1987. The assembly of blood clotting complexes on membranes. *TIBS (Trends Biochem. Sci.).* 12:229–233.

Mann, K. G., J. Elion, R. J. Butkowski, M. Downing, and M. E. Nesheim. 1981. Blood Clotting [23]. Prothrombin. *Methods Enzymol.* 80:286–302.

Marsh, H. C., Jr., P. Robertson, M. E. Scott, K. A. Koehler, and R. G. Hiskey. 1979. Magnesium and calcium ion binding to bovine prothrombin fragment 1. *J. Biol. Chem.* 254:10268–10275.

Mayer, L. D., M. J. Hope, and P. R. Cullis. 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta.* 858:161–168.

Myrmel, K. H., R. L. Lundblad, and K. G. Mann, 1976. Characteristics of the association between prothrombin fragment 2 and α-thrombin. *Biochemistry.* 15:1767–1773.

Nelsestuen, G. L. 1976. Role of γ-carboxyglutamic acid: an unusual protein transition required for the calcium-dependent binding of prothrombin to phospholipid. *J. Biol. Chem.* 251:5648–5656.

Nelsestuen, G. L., and M. Broderius. 1977. Interaction of prothrombin and blood-clotting factor X with membranes of varying composition. *Biochemistry.* 16:4172–4177.

Österberg, R., B. Sjöberg, P. Österberg, and J. Stenflo. 1980. Conformational change of human prothrombin induced by calcium ions: an x-ray scattering study. *Biochemistry.* 19:2283–2286.

Ploplis, V. A., D. K. Strickland, F. J. Castellino, 1981. Calorimetric evaluation of the existence of separate domains in bovine prothrombin. *Biochemistry.* 20:15–21.

Prendergast, F. G., and K. G. Mann. 1977. Differentiation of metal ion-induced transitions of prothrombin fragment 1. *J. Biol. Chem.* 252:840–850.

Privalov, P. L. 1979. Stability of proteins: small globular proteins. *Adv. Protein Chem.* 33:167–239.

Privalov, P. L. 1982. Stability of proteins: protein which do not present a single cooperative system. *Adv. Protein Chem.* 36:167–239.

Soriano-Garcia, M., C. H. Park, A. Tulinsky, K. G. Ravichandran, and E. Skrzypczak-Jankun, 1989. Structure of Ca^{2+} prothrombin fragment 1 including the conformation of the Gla domain. *Biochemistry.* 28:6805–6810.

Sturtevant, J. M. 1987. *Annu. Rev. Phys. Chem.* 38:463–488.

Szoka, F., F. Olson, T. Heath, W. Vail, E. Mayhew, and D. Papahadjopoulos. 1980. Preparation of unilammellar liposomes of intermediate size (0.1–0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. *Biochim. Biophys. Acta.* 601:559–571.

Tendian, S. C., and B. R. Lentz. 1990. Evaluation of membrane phase behavior as a tool to detect extrinsic protein-induced domain formation: binding of prothrombin to phosphatidylserine/phosphatidylcholine vesicles. *Biochemistry.* 29:6720–6729.

Wu, J. R., and B. R. Lentz. 1991. Fourier transform infrared spectroscopic study of Ca^{2+} and membrane-induced secondary structural changes in bovine prothrombin and prothrombin fragment 1. *Biophys. J.* 60:70–80.

Zwaal, R. F. A., and H. C. Hemker. 1982. Blood cell membranes and haemostasis. *Haemostosis.* 11:12–39.