Dynamic Features of Prothrombin Interaction with Phospholipid Vesicles of Different Size and Composition: Implications for Protein−Membrane Contact†

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ABSTRACT: The dynamics of prothrombin interaction with membrane vesicles of different size and composition was investigated to ascertain the impact of membrane surface characteristics and particle size on this interaction. Dissociation rates were highly sensitive to membrane composition and varied from about 20/s for membranes of 10% PS to 0.1/s for membranes of 50% PS. Overall affinity also varied by more than two orders of magnitude. Very small differences between prothrombin binding to SUV versus LUV were found. Association with large unilamellar vesicles (LUV of 115 nm diameter) was about 4-fold slower, when expressed on the basis of binding sites, than association with small unilamellar vesicles (SUV, 30 nm diameter) of the same composition. Both reactions proceeded at less than 25% of the collisional limit so that the differences were largely due to intrinsic binding properties. Vesicles of 325 nm diameter showed even slower association velocities. Dissociation rates from LUV were about 2-fold slower than from SUV. Again, these differences arose primarily from intrinsic binding properties. Dissociation conformed to a single first order reaction over a wide range of protein occupancy on the membrane. At very high packing density, the dissociation rate increased by about 2-fold. At equilibrium, prothrombin preferred binding to SUV over LUV by about 2-fold. This very small difference, despite substantial differences in phospholipid headgroup packing and hydrocarbon exposure, appeared inconsistent with an important role for protein insertion into the hydrocarbon region of the membrane. However, prothrombin−membrane interaction may arise from a series of interaction forces that have compensating features at equilibrium. The small differences in prothrombin binding to SUV versus LUV, together with differences in the number of protein binding sites per vesicle, were important to identify mechanisms of substrate delivery to the active site of the prothrombinase enzyme [Lu, Y., & Nelsestuen, G. L. (1996) *Biochemistry 35*, 8201−8209].

The association of prothrombin with phospholipid membranes is an integral part of blood coagulation. Other vitamin K-dependent proteins include factors X, IX, and VII and proteins C, S, and Z. These proteins contain homologous amino-terminal sequences of about 45 amino acids that include 9−13 γ-carboxyglutamate residues (Furie & Furie, 1988). This region of the protein binds calcium ions and supports interaction with phospholipid membranes. Specificity for binding to anionic phospholipids may aid in regulation of coagulation and localization of the blood clotting response to sites of cell damage and/or loss of membrane asymmetry (Bevers et al., 1982; Nelsestuen & Broderius, 1977; Zwaal et al., 1977).

Despite much study, structural features of the prothrombin−membrane contact remain speculative and somewhat contradictory. While the requirement for calcium provides a popular concept of calcium-bridging between protein carboxyls and anionic phospholipids, no direct evidence exists to support this model. The X-ray crystal structure shows seven calcium ions bound to prothrombin fragment 1[1] in a relatively planar configuration with a cluster of hydrophobic amino acid side chains projecting outward (Christiansen et al., 1994; Soriano-Garcia et al., 1992; Zhang & Castellino, 1994). Protein−membrane association has been suggested to occur via penetration of these hydrophobic side chains into the hydrocarbon region of the membrane to a depth that allows simultaneous calcium chelation by the protein and the membrane phospholipids (Christiansen et al., 1994; Colpitts & Castellino, 1994; Sunnerhagen et al., 1995; Zhang & Castellino, 1994). Hydrophobic interaction may help explain the lack of ionic strength impact on prothrombin−membrane association at saturating calcium levels (Resnick & Nelsestuen, 1980).

However, many questions remain. Three or four additional calcium ions, reported to associate with the prothrombin−membrane complex (Evans & Nelsestuen, 1994; Nelsestuen & Lim, 1977a; Sommerville et al., 1986), are not apparent. Penetration of protein through the headgroup region of the membrane should provide improved prothrombin interaction with membranes that provide greater access to the hydrocarbon region. Small unilamellar vesicles (SUV)[1] show

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[1] Abbreviations: prothrombin fragment 1, the amino-terminal 156 residues of prothrombin; PC, phosphatidylcholine isolated from egg; PS, phosphatidylserine isolated from bovine brain; BODIPY-PA, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacenepentanonyl)-1-hexandecanoyl-*sn*-glycero-3-phosphate; NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazole-L-α-phophatidylethanolamine; SUV, small unilamellar vesicles which have measured diameters of 25−30 nm; LUV, large unilamellar vesicles produced by extrusion technique and with average measured diameters of 110−118 nm; HUV, huge unilamellar vesicles of about 330 nm diameter.

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phospholipid surface areas of 74 nm^2 on the outer leaflet (Huang & Mason, 1978), compared with only 55 nm^2 for large unilamellar vesicles [LUV (Deamer & Bangham, 1976)]. Proteins that penetrate into the hydrocarbon region of the membrane, such as cytrochrome b_5 (Greenhut et al., 1986) and complement proteins (Silversmith & Nelsestuen, 1986), can show preferences of over 100-fold for binding to SUV. Even blood clotting factor V, whose penetration into the membrane may be limited, showed significant preference for binding to SUV (Abbott & Nelsestuen, 1987). However, equilibrium binding constants for interaction of prothrombin and other vitamin K-dependent proteins with SUV (Nelsestuen & Broderius, 1977), phospholipid monolayers spread at the air−water interface (Mayer et al., 1983), planar phospholipid bilayers (Andree et al., 1994), and supported monolayers (Pearce et al., 1992; Pearce et al., 1993) appear to be quite similar at constant composition. In some cases, prothrombin association with phospholipid monolayers occurred without increase in monolayer surface pressure (Mayer et al., 1983). These properties suggest interaction with only the phospholipid headgroup.

In contrast to the similarity of equilibrium binding constants, association and dissociation rate constants for SUV and planar monolayers varied by almost two orders of magnitude [1×10^7 M^{-1} s^{-1} and 4 s^{-1} for SUV (Wei et al., 1982) and 1×10^5 M^{-1} s^{-1} and 0.02−0.5 s^{-1} for monolayers (Pearce et al., 1992)]. Dissociation rates from supported monolayers was heterogeneous (Pearce et al., 1992). Previous studies have obtained rate constants from a combination of one velocity measurement [either k_{assn} (Wei et al., 1982) or k_{dissn} (Pearce et al., 1992)] and estimation of the other from the equilibrium binding constant. While a recent study examined both rate constants directly, k_{dissn} was measured by displacement by another protein (Kung et al., 1994), a technique that does not result in variation of protein density on the membrane. Slight heterogeneity of equilibrium binding to SUV has been reported as prothrombin reached high packing density on the membrane surface (Wei et al., 1982; Dombrose et al., 1979). Other proteins show extensive heterogeneity as a function of protein packing density on the membrane (Abbott & Nelsestuen, 1987; Bazzi & Nelsestuen, 1991b). Heterogeneous behavior may be important if a minor population of protein was responsible for important aspects of prothrombin function.

Major differences in phospholipids of various types have also been suggested by thermodynamic properties and enzyme kinetics. Direct titration calorimetry showed that prothrombin interaction with SUV was almost entirely enthalpic with a ΔH of about −7 kcal/mol while interaction with LUV was almost entirely entropic (Plager & Nelsestuen, 1994). K_M values for prothrombinase varied from 200 nM for SUV to 40 nM for LUV (Giesen et al., 1991) of the same composition and were 3−6 nM for phospholipid bilayers supported on planar surfaces (Billy et al., 1995; Giesen et al., 1991). These very large differences suggest the possibility that comparison of equilibrium binding constants may overlook some of the most important aspects of prothrombin−membrane structure that also regulate function.

This study investigated dynamic features of prothrombin interaction with two types of phospholipids, SUV and LUV. These were studied by similar, newly developed methods which provided high sensitivity and precision so that small differences were detectable. Rate constants were surprisingly homogeneous, changing by only 2-fold at high protein packing density on the membrane. Differences between SUV and LUV consisted of lower association and dissociation rate constants for LUV and overall affinity that preferred SUV by about 2-fold. These precise comparisons suggested little importance of protein insertion into the hydrocarbon region of the phospholipid bilayer. The studies also provided a valuable comparison to enzyme kinetic properties (Lu & Nelsestuen, 1996).

MATERIALS AND METHODS

Materials. Bovine brain phosphatidylserine (PS) and *N*-4-nitrobenzo-2-oxa-1,3-diazole-L-α-phophatidylethanolamine (NBD-PE) were purchased from Avanti Polar Lipids, Inc., egg phosphatidylcholine (PC) was purchased from Sigma Chemical Co., and 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacenepentanonyl)-1-hexandecanoyl-*sn*-glycero-3-phosphate (BODIPY-PA) was purchased from Molecular Probes, Inc. All phospholipids were of high purity (>98%, suppliers' estimate). Polycarbonate filters (0.1 μm diameter) were purchased from Nucleopore Corp. (Costar Co.). Lissamine Rhodamine sulfonyl chloride was purchased from Molecular Probes, Inc. Other chemicals were from Sigma Chemical Co. and were of the highest grade available. Bovine prothrombin and prothrombin fragment 1 were prepared and quantitated by published methods (Nelsestuen, 1984).

Protein Labeling. Prothrombin and fragment 1 were labeled with lissamine rhodamine essentially as described by Chen (1969). Lissamine rhodamine sulfonyl chloride was dissolved in dimethyl sulfoxide (10 mg/mL) immediately before use and was added dropwise to a solution of prothrombin (2 mg/mL) in 0.1 M $NaCO_3$ buffer to a final level of 25 μL of dye solution per mL of protein. The solution was then stored at 5 °C and stirred for 4 h. Free dye was removed by gel filtration on a column of Sephacryl S-300 (1.5 × 30 cm). Dye incorporated per protein is difficult to quantitate precisely (Chen, 1969). An estimate was obtained from the absorbance of the protein solution at 550 nm and the extinction coefficient for the free dye (1.3×10^4 $M^{-1}cm^{-1}$ at 550 nm (Taylor et al., 1980)). A typical preparation gave 4.5 dye molecules per prothrombin molecule. Thrombin digestion of the labeled prothrombin followed by separation of fragment 1 and intermediate 1 on ion exchange chromatography (Heldebrant & Mann, 1973) showed that 64% of the fluorescence intensity eluted with the intermediate 1 portion of prothrombin. Fragment 1 was labeled directly with 50 μL of the dye solution per mL of protein. About 2.5 dye molecules were incorporated per fragment 1 molecule.

Vesicle Preparations. Published techniques were used. BODIPY-PA was dissolved in chloroform. All other phospholipids were provided in chloroform/methanol (95:5). The components were mixed in the desired ratios and the organic solvent was evaporated by a stream of nitrogen. The sample was then placed under high vacuum for 1 h. The dried phospholipids were dispersed in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.5) at a final concentration of 2.0 mg/mL. Large unilamellar vesicles (LUVs) were prepared by multiple freeze−thaw cycles and extrusion through polycarbonate membranes with a pore size of 100 nm (Hope et al., 1985). They were dialyzed against Tris buffer containing

100 mM NaCl to produce spherical shapes. Huge unilamellar vesicles (HUV) were prepared by ether injection (Deamer & Bangham, 1976). The dried phospholipids were dissolved in ether solution (1.6 mg/mL) which was slowly injected (0.4 mL/min) into Tris buffer (100 mM NaCl) that was maintained at 60 °C. The vesicles were then extruded through a 600 nm polycarbonate filter and gel-filtered on a column of Sepharose 4B (Pharmacia Fine Chemicals). The vesicles eluting at the exclusion volume of the column were pooled. Small unilamellar vesicles (SUVs) were prepared as described previously (Huang, 1969) by sonication followed by gel filtration chromatography on Sepharose 4B.

Phospholipid vesicle sizes were determined by dynamic light scattering as described previously (Bloomfield & Lim, 1978). SUV of 50% PS gave average diameters of 27 nm while SUV of 25% PS gave 26 nm. LUV of both types gave average diameters of 118 nm. The molecular weights were calculated from these values, molecular weights of 760 for PC and 810 for PS, and the reported surface areas for phospholipids in SUV and LUV. Surface areas per phospholipid were 0.74 and 0.61 nm^2 for the outer and inner leaflets of SUV (Huang & Mason, 1978), respectively, and 0.55nm^2 for both leaflets of LUV (Deamer & Bangham, 1976). Membrane thickness is 3.7 nm for SUV and 5.0 nm for LUV. The molecular weights were 4.1×10^6 for SUV of 50% PS, 3.5×10^6 for SUV of 25% PS and 1.15×10^8 for LUV of either composition. These molecular weights for SUV correlated closely with earlier estimates obtained by light scattering intensity measurements [3.2×10^6 for SUV of 20% PS and 3.7×10^6 for SUV of 40% PS (Wei et al., 1982)]. SUV of 10% PS gave molecular weights of 4×10^6.

Equilibrium Binding of Prothrombin to Vesicles. Maximum protein binding of proteins to SUVs was monitored by light scattering at 320 nm as detailed elsewhere (Nelsestuen & Lim, 1977). A FluoroMax (JY/Spex Instruments SA, Inc.) fluorescence spectrophotometer was used to measure 90° light scattering intensity. The maximum protein bound to phospholipid was 0.5 (g/g) for SUV of 10% PS, 1.2 (g/g) for 25% PS, and 1.4 (g/g) for 50% PS. These values, together with vesicle and protein molecular weights gave 30, 58, and 80 prothrombin binding sites per SUV of 10, 25, and 50% PS, respectively. These values correlated very closely to earlier reports [30, 57, and 72 for SUV of 10, 20, and 40% PS, respectively (Wei et al., 1982)]. For LUV, the maximum prothrombin bound could not be determined directly by light scattering. Capacity was estimated by the amount of prothrombin bound to SUV and the relative amount of phospholipid on the outer leaflet of LUV (67% of the phospholipid is on the outer leaflet for SUV and 50% for LUV). This corresponded to 1430 and 1660 sites for LUV of 25 and 50% PS, respectively. LUV of 10% PS gave 560 binding sites per vesicle of 107 000 kDa.

Competition Binding to Different Vesicles. The relative distribution of labeled prothrombin between different vesicle preparations was determined by competition. Fluorescence energy transfer between lissamine Rhodamine-labeled prothrombin and fluorescent vesicles (20 μg of LUV, PS/PC/BODIPY-PA, 50/49/1) was measured with excitation at 500 nm and emission at 600 nm. Calcium (2 mM) and lissamine Rhodamine-labeled prothrombin (3 μg/mL) were added to mixtures of unlabeled vesicles [PL] and the labeled LUVs [PL*]. The fluorescence intensity due to energy transfer was measured before and after addition of excess EGTA (4 mM). The difference between these two readings (ΔF) was proportional to the amount of protein bound to the labeled vesicles. The competitive prothrombin-binding capability of the different vesicles (C) was defined by eq 1 where ΔF_o

$$C = (\Delta F_o/\Delta F_n - 1)/([PL]/[PL^*]) \quad (1)$$

is the fluorescence change for this experiment in the absence of unlabeled vesicles and ΔF_n is the fluorescence change obtained in the presence of unlabeled phospholipids. Unlabeled LUV containing 50% PS gave a C value of about 1.0.

Stopped-Flow Measurements. Fast kinetics of protein binding to, and dissociation from, vesicles were monitored by fluorescence energy transfer and/or light scattering using a 4800C Spectrofluorometer (SLM. Aminco) with a stopped-flow attachment (Mili-Flow, SLM. Aminco) described in detail elsewhere (Lu et al., 1995). Briefly, equal volume syringes were filled with appropriate solutions, and about 40 μL from each syringe was used per injection. The dead time of the instrument was about 6 ms as measured by the fluorescence reaction of pyranine with bovine carbonic anhydrase as described by the manufacturer. A typical experiment for estimation of protein−membrane association used a solution of phospholipid vesicles in one syringe and prothrombin in the other. For a typical dissociation experiment, a solution containing labeled vesicles and labeled prothrombin was placed in one syringe. Unlabeled SUVs at 10−20 times the concentration of labeled vesicles and composed of the same or a higher percentage of PS was placed in the other. The unlabeled vesicles were sufficient to capture all of the free prothrombin and create irreversible dissociation from the labeled vesicles. For both association and dissociation, the final fluorescence or light scattering intensity was obtained 2−5 min after the reaction. Data were fit to this final value. Fluorescence changes obtained by mixing vesicles with buffer in the stopped-flow were measured as a background to detect processes such as photobleaching. These changes were small and easily subtracted from experimental data. Inclusion of bovine serum albumin (1 mg/mL) in all buffers eliminated these small background changes entirely. Rate constants obtained in the presence and absence of bovine serum albumin were indistinguishable.

The dissociation process was well fit by a single first order rate constant and was independent of the concentration of unlabeled SUV used (range above). Rate constants were also independent of the phospholipid composition of the unlabeled vesicles (25 or 50% PS), as long as they bound prothrombin with adequate affinity to prevent significant reassociation with the first vesicle population. The dissociation reaction was also measured by addition of a large excess of unlabeled prothrombin (at least 20-fold excess). Three-fold variation of the amount of prothrombin indicated that this excess was sufficient to allow analysis of dissociation as an irreversible reaction.

Protein binding and dissociation processes were primarily monitored by fluorescence energy transfer from NBD or BODIPY (donor) on the membrane to the lissamine Rhodamine (acceptor) on the protein. The excitation wavelength was 460 nm (for NBD) or 500 nm (for BODIPY)

and emission was measured through a 600 ± 10 nm bandpass filter (Corion Corp.).

While most of the data shown in this study were obtained with BODIPY-labeled membranes, NBD-labeled membranes gave similar results. Fluorescence intensity due to energy transfer was proportional to the amount of protein bound to the membrane, and the change of intensity was analyzed directly as an indicator of reaction progress. At high protein density on the membrane, a small self-quenching of the lissamine Rhodamine was detected. This did not have significant impact on the results or conclusions of this study.

Association kinetics were also measured by light scattering intensity at 90° to the incident light and at a wavelength of 320 nm (Wei et al., 1982). The amount of protein bound to the membrane was estimated from M_2/M_1, the molecular weight of the protein−vesicle complex divided by that of the vesicles alone. This ratio is proportional to the square root of light scattering intensity and refractive index increments of the two species (Nelsestuen & Lim, 1977b). However, at low changes of mass (less than 25%), the progression of the light scattering intensity change was very nearly equal to the progression of mass change (M_2/M_1). For these situations, light scattering intensity change was usedas an indicator of reaction progress. Error induced by this simplification was less than than signal to noise ratios in the kinetics experiments shown [e.g., see Lu et al. 1995)].

All data shown are the average of of at least three measurements with the same set of samples. The deviations for replicate experiments with the same reagents were approximately equal to signal to noise ratios. In all cases, prothrombin had been preincubated with calcium for a time that was adequate to allow completion of its calcium-dependent conformational change.

Data Analysis. The interaction between prothrombin and vesicles can be analyzed as a bimolecular reaction between protein and binding sites on the vesicle surface (Wei et al., 1982). The relationship in eq 2 assumes that all sites were

$$\mathrm{P} + \mathrm{PL}_{\mathrm{site}} \underset{k_{\mathrm{dissn}}}{\overset{k_{\mathrm{assn}}}{\rightleftharpoons}} \mathrm{P{-}PL} \qquad (2)$$

identical and independent and that k_{assn} and k_{dissn} were constant throughout the reaction. The results (below) suggest that these conditions were very nearly met for the reaction, except at very high protein packing density.

At low protein/vesicle ratios (<0.25, w/w), the reaction could be treated as a reversible pseudo-first-order reaction. Since the fluorescence and light scattering intensity changes were analyzed directly as indicators of reaction progress (see above), the apparent pseudo-first-order rate constants (k_{app}) were obtained by fitting the reaction curves with a first order reaction using the computer program KaleidaGraph. The kinetic parameters for this type of reaction are described by (Fierke & Hammes, 1995).

$$k_{\mathrm{app}} = k_{\mathrm{assn}}[\mathrm{site}] + k_{\mathrm{dissn}} \qquad (3)$$

k_{assn} is the second order association rate constant expressed on the basis of binding site concentration [site], and k_{dissn} is the first order dissociation rate constant. Both k_{assn} and k_{dissn} can be obtained from k_{app} values measured at different concentrations of binding sites. While dissociation rate constants obtained in this manner were comparable to those measured directly, only the latter are reported below. Direct measurements by fluorescence methods gave standard deviations for replicate measurements of 5−20%.

Second order association rate constants expressed on the basis of vesicle concentration can be obtained from $k_{assn}N$, where N is the number of binding sites per vesicle. Collisional efficiency was estimated by divided $k_{assn}N$ by k_{coll}, the rate constant for collision between protein and vesicles. Spherical shape was assumed for estimation of k_{coll} (Smoluchowski, 1917).

$$k_{\mathrm{coll}} = 4\pi N_{\mathrm{A}} D r/1000 \qquad (4)$$

N_A is Avogadro's number, D is the sum of the vesicle and protein diffusional coefficients (effectively equal to that of the protein), and r is the sum of the vesicle and protein radii (effectively that of the vesicles). The diffusion coefficient for prothrombin was 6.1×10^{-7} cm^2/s (Wei et al., 1982).

Other Methods. Protein concentrations were determined according to Bradford (1976), and phospholipid concentration was determined from organic phosphate measured according to Chen et al. (1956). A 25:1 phospholipid to phosphorus weight ratio was used. Unless indicated, the buffer system used throughout this study was 50 mM Tris and 100 mM NaCl, pH 7.5, and the calcium concentration was 2 mM. The temperature was 25 ± 1 °C unless otherwise specified.

RESULTS

Association of Prothrombin with Membrane Vesicles. Lissamine Rhodamine-labeled prothrombin showed membrane binding characteristics that were indistinguishable from those of unmodified protein. Prothrombin binding to vesicles containing fluorescent phospholipids was also indistinguishable from binding to unlabeled vesicles. Examples of steady-state membrane binding by labeled and unlabeled prothrombin are shown in Figure 1A. The kinetics of prothrombin binding, measured by light scattering intensity changes or by fluorescence energy transfer to NBD-PE in the membrane, were superimposible (Figure 1B). Comparisons of labeled and unlabeled prothrombin gave similar correlations (not shown). BODIPY-PA was used for most measurements shown below.

Rate constants were independent of the method of data analysis. Wei et al. (1982) fit association reactions with the ratio of association and dissociation rate constants that were obtained from equilibrium binding measurements. A simpler method of analysis was applied for most studies in this report. Pseudo-first-order rate constants (k_{app}), determined at varying protein/phospholipid ratios, provided a linear relationship (Figure 2, inset) from which k_{assn} could be obtained using eq 3.

An exception to the pseudo-first-order condition was membranes of 10% PS. In this case, k_{dissn} was the dominant term in eq 3 and k_{assn} could not be obtained with high accuracy. For these membranes, rate constants (Table 1) were estimated by the approach of Wei et al. (1982) but with use of k_{dissn} determined by direct measurement (Table 1). Overall, association rate constants for SUV (Table 1) agreed very well with previously published values (Wei et al., 1982).

Fluorescence energy transfer allowed direct comparison of association of prothrombin with vesicles of different sizes. Figure 3 shows typical results. While the rate constants for the interactions were a substantial fraction of the collisional

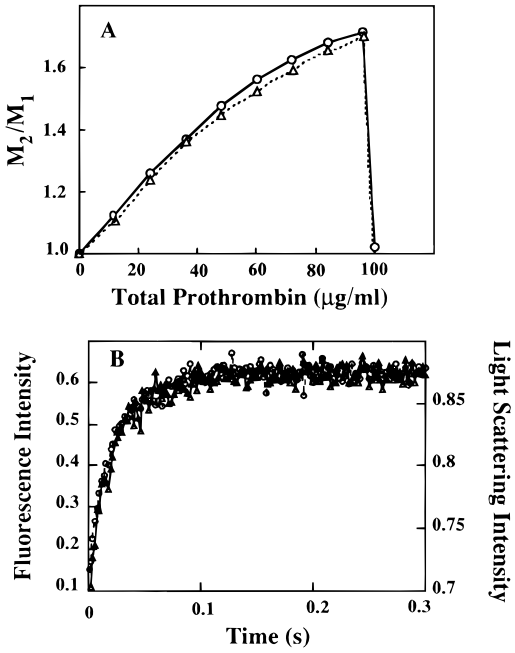

FIGURE 1: Association of prothrombin with vesicles. (Panel A) Equilibrium protein titrations are shown for association of unlabeled (○) and labeled (△) prothrombin with SUVs (80 μg/mL, PS/PC/NBD-PE, 25/72/3). The calcium concentration was 2 mM. The interaction was measured by light scattering at 320 nm. M_2 and M_1 are the molecular weights of the vesicle−protein complex and vesicles alone, respectively. Addition of EGTA returned the signals to their initial values (final data points on the right). (Panel B) Kinetics of prothrombin association with vesicles. The reactions were initiated by rapid mixing of an equal volume of lissamine Rhodamine-labeled prothrombin solution (40 μg/mL after mixing) with vesicles (160 μg/mL of PS/PC/NBD-PE, 25/72/3 after mixing) in the stopped-flow. Both solutions contained 2 mM calcium. The interaction was measured by both light scattering (320 nm, ○) and fluorescence energy transfer (excitation at 460 nm, emission at 600 ± 10 nm, △).

limit (Table 1), a process that provides a basis for slower overall reaction with large vesicles (Abbott & Nelsestuen, 1987), several lines of evidence suggested that most of the differences arose from intrinsic site-binding behavior. For example, activation energies for the interactions were much higher than expected for a collisionally efficient process (Table 1). Prothrombin showed a 2-fold preference for interaction with SUV over LUV (Table 1). A further small change occurred with HUV.

Dissociation Rate Behavior. Figure 4A shows typical results for dissociation of prothrombin from SUV of three

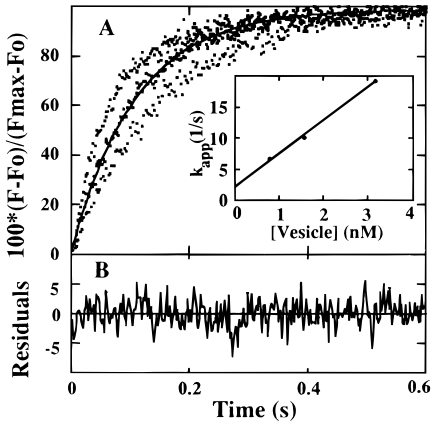

FIGURE 2: Dependence of association rate constants on vesicle concentration. The binding reactions were measured at fixed protein concentration (20 μg/mL of lissamine Rhodamine-labeled prothrombin after mixing) and varied vesicle (LUVs, PS/PC/BODIPY-PA, 25/75/1) concentrations. From left to right the data represent 320, 160, and 80 μg/mL (after mixing), respectively. Fluorescence energy transfer (excitation at 500 nm, emission at 600 ± 10 nm) was used to detect the protein−membrane complex. F_o, F, and F_{max} represent the fluorescence intensity due to energy transfer at the beginning, at time t and at the end of the reaction (2 min after mixing), respectively. (Inset) The pseudo-first-order rate constants (k_{app}) obtained are plot versus vesicle concentration. Rate constants obtained from the data and eq 3 at all phospholipid were averaged to obtain the second order rate constants reported in Table 1.

compositions. For membranes of all compositions and sizes and for all prothrombin/vesicle ratios used, the data were fit very well by single first order rate constants (e.g., see residuals, Figure 4B). The several control experiments outlined in Materials and Methods suggested that these approaches measured the prothrombin−membrane dissociation rate constant and were not influenced by the fluorescent probes or by direct protein transfer during vesicle−vesicle collisions. Similar rate constants were obtained for 0.25−1.0 initial ratios (w/w) of prothrombin to membrane.

Previous studies suggested small changes in equilibrium binding properties as the membrane approached saturation with respect to prothrombin (Wei et al., 1982). To examine this more closely, dissociation was measured after addition of an excess of unlabeled prothrombin. In this case, the labeled prothrombin dissociated from a membrane that was always saturated with protein; the lowest affinity interactions should be emphasized. Again, the results were fit well by a single first order rate constant that differed by only about

Table 1: Kinetic Properties of Prothrombin−Membrane Interaction

membrane composition	size (nm)	N	k_{assn}[a] ($\times 10^{-6}$ s^{-1} M^{-1})	k_{dissn} (1/s) excess PL[b]	k_{dissn} (1/s) excess PT[c]	K_D (μM)	binding competition[d]	Nk_{assn} ($\times 10^{-9}$ s^{-1} (M^{-1})	collisional efficiency[e]	E_a (kcal/mol)[f] k_{assn}	E_a (kcal/mol)[f] k_{dissn}
10% PS	26	30	15.3	24		1.6		0.45	7%		
	118	560	4.0	17		3.9		2.2	8%		
25% PS	27.5	58	17.6 ± 1.0	1.9	4	0.11	4 ± 0.1	1.2	18%	11.2	15.4
	119	1420	4.3 ± 0.5	1	2.8	0.23	1	6.1	22%	12.0	15.7
	328	13 000	1.1	0.9		0.86		27	34%		
50% PS	26	80	18.0 ± 0.7	0.15	0.28	0.008	60 ± 19	1.4	22%		
	118	1660	4.4 ± 0.5	0.09		0.02	18 ± 8	7.3	26%		
25% PS (fragment 1)	27.5	58	16 ± 1.4	1.7		0.11		0.9			

[a] Values expressed per binding site. Standard deviations for k_{assn} determined at different phospholipid concentrations (Figure 2, inset). [b,c] Measured by mixing with excess unlabeled phospholipid (PL) or unlabeled prothrombin (PT) at zero time (Materials and Methods). [d] Determined as in Figure 5 and eq 1. [e] Collisional efficiency = Nk_{assn}/k_{coll}. [f] Rate constants were determined at five temperatures between 10 and 30 °C. Correlation coefficients of the Arrhenius plots were R > 0.99.

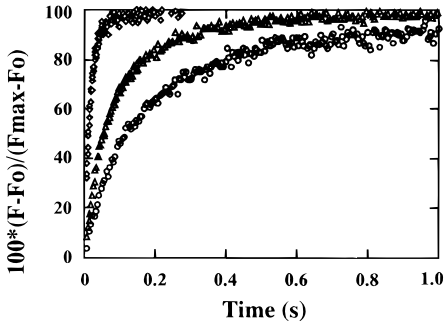

FIGURE 3: Dependence of association rate constants on vesicle radius. The binding reactions were measured with labeled prothrombin (40 μg/mL after mixing) and vesicles (200 μg/mL after mixing, PS/PC/BODIPY-PA, 25/75/1) of different diameters. The curves shown from left to right were for vesicles of 28, 114, and 326 nm. Fluorescence intensities are as described in Figure 2. The pseudo-first order-rate constants obtained from these curves were 61, 13, and 6.6 s^{-1}, respectively.

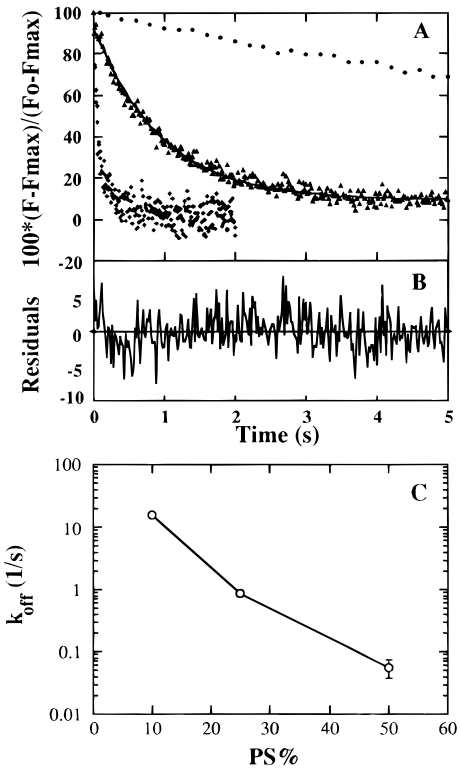

FIGURE 4: Dissociation of prothrombin from LUVs. (Panel A) A solution of lissamine Rhodamine−prothrombin (40 μg/mL) and BODIPY-PA-containing vesicles (80 μg/mL) was mixed with an equal volume containing unlabeled SUV (900 μg/mL, PS/PC, 50/50) and the fluorescence signal was monitored. F, F_{max}, and F_o are the fluorescence intensity due to energy transfer at the time of measurement, at maximum time (≥2 min after mixing) and at zero time, respectively. From left to right, the results were from vesicles containing PS/PC/BODIPY-PA at ratios of 10/90/1, 25/75/1, and 50/50/1, respectively. These curves were all fit as first order reactions. The solid line shows the fit of the middle curve. (Panel B) Residuals between the experimental data and the fitted curve from panel A. (Panel C) The first order dissociation rate constants from experiments such as those in panel A are plotted as a function of PS composition of the membranes. The concentration of PC was varied to accommodate the PS composition. The results represent the averages and standard deviations from at least three independent measurements.

2-fold from those estimated by addition of excess unlabeled vesicles (Table 1). Thus, this interaction displayed a low sensitivity to the presence of other prothrombin molecules.

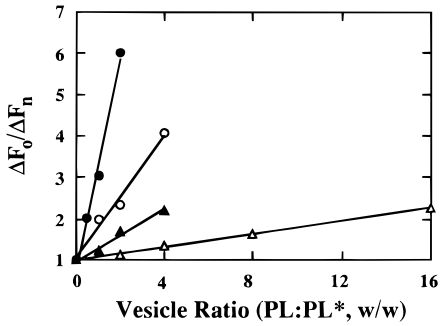

FIGURE 5: Competition for binding of prothrombin to different vesicles. Binding between lissamine Rhodamine−prothrombin and labeled vesicles (20 μg/mL of LUV containing PS/PC/BODIPY-PA, 50/49/1) was monitored by fluorescence energy transfer (excitation at 500 nm and emission at 600 nm). To assess competition, varied amounts of unlabeled vesicles (PL) were mixed with these labeled phospholipids (PL*) at the ratios of [PL]/[PL*] (w/w) shown. The slope of the line is then equal to C (eq 1). Calcium (2 mM) and labeled prothrombin (3 μg/mL) were then added, and the fluorescence intensity was measured. Excess EGTA (4 mM) was added, and the fluorescence intensity was measured again. ΔF_o is the difference between these fluorescence intensities in the absence of unlabeled vesicles, and ΔF_n is the difference in the presence of unlabeled vesicles at the ratios shown. The unlabeled vesicles were SUVs (●, PS/PC, 50/50), LUVs (○, PS/PC, 50/50), SUVs (▲, PS/PC, 25/75), and LUVs (△, PS/PC, 25/75).

The results were in good agreement with earlier studies of membranes with low PS content (<25% PS). In some cases, the 2-fold range in dissociation rate for SUV vs LUV made an important contribution to enzyme kinetic behavior (Lu & Nelsestuen, 1996).

An important new finding was the slow dissociation of prothrombin from vesicles of high PS content. Earlier studies suggested a relatively constant dissociation rate for membranes above 25% PS (Wei et al., 1982). However, those studies did not observe dissociation rates directly but obtained them from equilibrium constants and modeling of association reactions. Equilibrium constants, obtained from the concentration of free protein, become less accurate as the amount of free protein declines. The earlier values also utilized different calcium concentrations. High calcium may lower the affinity of prothrombin for the membrane, a phenomenon that was readily detected for protein S (Schwalbe et al., 1989). In any event, the values for prothrombin dissociation from membranes of 50% PS at 2 mM calcium provided important information that allowed more accurate interpretation of enzyme kinetics under the same conditions (Lu & Nelsestuen, 1996).

Competition experiments verified the relative affinities of prothrombin that were obtained from association and dissociation rate constants. The experiments in Figure 5 show competitive binding of prothrombin to fluorescent vesicles (LUV of 50% PS) in the presence of unlabeled vesicles. A value of 1.0 was assigned to LUV of 25% PS, the other membranes (LUV of 50% PS, SUV of 25% PS, and SUV of 50% PS) showed relative binding capacities of about 18, 4, and 60, respectively. These competition values agreed quite well with the capacity and binding affinities for these respective membranes, determined by direct measurement (Table 1).

Thermodynamic Behaviors. The activation energies for association reactions were 11.2 and 12 kcal/mol for SUV and LUV of 25% PS, respectively (Table 1). Activation energy is highly dependent on both phospholipid content and

calcium concentration (Wei et al., 1982), so that these values applied only to the specific conditions used. These activation energies indicated that the reactions were limited by intrinsic properties of the site binding events rather than by collisional events. Collision-efficient reactions should show an activation energy of about 4.6 kcal/mol.

The activation energy for dissociation was 15.4 and 15.7 kcal/mol for SUV and LUV of 25% PS, respectively (Table 1). This was the first estimate of activation energy for prothrombin−membrane dissociation obtained by direct measurement. While the major conclusion from these data was that prothrombin showed little difference in binding to LUV versus SUV, selectivity had a significant influence on enzyme kinetic properties (Lu & Nelsestuen, 1996).

Kinetic Behavior of Fragment 1. Normally, the membrane-binding region of prothrombin is assumed to be located in the amino-terminal fragment 1 region of prothrombin (residues 1−156) which contains all of the γ-carboxyglutamate residues. Most of the membrane binding ability may reside in the amino-terminal 45 residues. Amino acids in the second disulfide loop of prothrombin (residues 48−61) make a small contribution to tighter calcium binding since the amino terminal 45 residues of prothrombin requires higher calcium for interaction with membranes than does fragment 1 (Schwalbe et al., 1989). Thrombin cleavage of protein S at residues 52 and 70 also produces reduced calcium binding ability (Dahlback, 1983; Schwalbe et al., 1989). However, some studies have suggested that portions of prothrombin that are C-terminal to fragment 1 also contribute to membrane binding. Scanning calorimetry of prothrombin suggested a difference in denaturation patterns of the C-terminal region of prothrombin when it was associated with membranes versus when it was free in solution (Lentz et al., 1991, 1994). Equilibrium measurements often suggest that fragment 1 has a slightly lower affinity for membranes than does prothrombin. While estimation of affinity from equilibrium measurements can pose problems if a portion of the free protein is nonfunctional, dissociation rate constants should be insensitive to this problem.

The rate constants for dissociation of fragment 1 from membranes were virtually identical to those for prothrombin (Table 1). These precise comparisons indicated that fragment 1 contained the entire membrane-binding portion of the protein and that contributions by the C-terminal region of prothrombin were exceedingly small. Similarity also extended to association rate constants (Table 1).

DISCUSSION

This study concentrated on one vitamin K-dependent protein, a single calcium concentration, and a small number of membrane structures in order to obtain precise physical measurements that might suggest features of the prothrombin−membrane interaction site and which could be used to determine enzyme kinetic mechanisms (Lu & Nelsestuen, 1996). Phospholipid structure in SUV and LUV differ significantly. The greater surface curvature and area per headgroup in SUV provides a higher free energy of about 2 kcal/mol phospholipid (Suurkuusk et al., 1976). Any process that relieves this surface strain should be energetically favorable. For example, proteins or peptides that interact with the hydrocarbon region of the membrane should reduce surface strain and prefer interaction with SUV. While many proteins show great preference for interaction with SUV over LUV (Abbott & Nelsestuen, 1987; Greenhut et al., 1986; Silversmith & Nelsestuen, 1986), the degree of preference displayed by prothrombin was so low that precise methods were needed for its detection. A 2-fold greater affinity of prothrombin for interaction with SUV corresponded to about 0.6 kcal/mol prothrombin, much less than the difference in surface free energy of a single phospholipid in SUV vs LUV.

In the first version of this paper, an attempt was made to reconcile the physical properties documented in this study with membrane-binding interactions suggested by the X-ray crystal structure, which consist of insertion of amino acid side chains into the hydrocarbon region of the membrane. A reviewer correctly criticized this as contributing to "...unclarities...". In fact, the properties documented in this study provide little support for prothrombin−membrane binding by insertion of amino acid side chains into the hydrocarbon region of the membrane.

Because of this problem, another study was initiated which has found that proline 22 is in the cis configuration in the membrane-binding conformation of bovine prothrombin (Evans & Nelsestuen, 1996). The X-ray crystal structure shows this proline in a trans configuration (Soriano-Garcia et al., 1992) so there may be no need to reconcile the X-ray crystal structure with the properties outlined in this study.

An interesting comparison can be made between vitamin K-dependent proteins and the annexins, another family of proteins that bind to membranes in a calcium-dependent manner. Originally, calcium binding measurements and the number of calcium ions bound to the protein in the crystal were quite divergent, with many more sites suggested by direct binding measurements. However, the most recent studies show 10 calcium ions per annexin molecule in both direct binding studies (Evans & Nelsestuen, 1994) and in protein that was co-crystalized with calcium and phosphoserine (Swairjo et al., 1995). Annexin−membrane interaction is achieved by calcium bridging between partial binding sites on the protein and on the membrane. A minor hydrophobic contribution may arise from hydrophobic "flaps", widely separated side chains of one tryptophan, two alanines, and one leucine (Swairjo et al., 1995). The large number of bridging calcium ions is consistent with low calcium affinity of the free protein, with unusual behavior referred to as "sequential binding", and with unusually high affinity of the protein for the membrane (Bazzi & Nelsestuen, 1991b). Thus, calcium and membrane-binding properties of annexins appear to be entirely consistent with the complete X-ray crystal structure. It is possible that a similar structural theme, involving three or four bridging calcium ions that are not observed on the membrane-free protein (Evans & Nelsestuen, 1994), may exist for prothrombin. Full determination of similarities to annexins must await determination of the correct structure of a vitamin K-dependent protein structure in its membrane-bound state.

The small degree of variation in association and dissociation rate constants as a function of membrane occupancy by protein was somewhat surprising, although it agreed with equilibrium binding studies (Wei et al., 1982). This suggested that prothrombin had little influence on membrane

structure. Protein density on the membrane has a major impact on factor V (Abbott & Nelsestuen, 1987) and annexins (Bazzi & Nelsestuen, 1991b). One source of this anticooperativity for the annexins appeared to be clustering of acidic phospholipids (Bazzi & Nelsestuen, 1991a). Lack of a similar effect for prothrombin suggested that little clustering occurred (Tendian & Lentz, 1990). Identical behavior of prothrombin and fragment 1 indicated that the entire membrane-binding region of prothrombin is located in the amino terminal 156 residues.

At constant calcium, prothrombin−membrane binding showed high cooperativity with respect to PS in the membrane. Increasing the PS content 5-fold, from 10 to 50% PS, resulted in a 140-fold decrease in k_{dissn} and a negligible increase in k_{assn}. The change was relatively constant with a 13-fold decrease in k_{dissn} from 10 to 25% PS (2.5-fold change in PS) and a 9-fold decrease in k_{dissn} from 25 to 50% PS (2-fold change in PS). This cooperativity undoubtedly arose from several sources. Attraction of multivalent metal ions to the membrane should show cooperativity, calcium binding to prothrombin is also cooperative, and protein contact with the membrane may involve more than one PS molecule. While it was apparent that prothrombin binding was highly influenced by PS content, assigning cooperativity to a specific event may not be possible.

Although small, the differences in interaction of prothrombin with SUV and LUV may be part of a continuum that extends to supported planar bilayers. The dynamics of protein dissociation from supported bilayers differed greatly from those of vesicles of the same composition [k_{dissn} of 0.5 to 0.02/s (Pearce et al., 1992)], despite K_D values that were similar. Thus, k_{dissn} varied in the order: SUV > LUV > planar bilayers. Efficacy of function in prothrombinase, as reflected by substrate concentration needed to reach half maximum velocity, proceeded in the same sequence (SUV > LUV > planar bilayers). It is possible that surface structures (other than composition) which impact on k_{dissn} may be important for enzyme action.

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