Hawkes, R., Niday, T., & Gordon, J. (1982) *Anal. Biochem. 119*, 142-147.
Laemmli, U. K. (1970) *Biochemistry 9*, 4620-4626.
Lotscher, H.-R., deJong, C., & Capaldi, R. A. (1984) *Biochemistry 23*, 4134-4140.
Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206-210.
Matsuno-Yagi, A., Yagi, T., & Hatefi, Y. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7550-7554.
Mendel-Hartvig, J., & Capaldi, R. A. (1991) *Biochemistry 30*, 1278-1284.
Penefsky, H. S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1589-1593.
Richter, M. L., & McCarty, R. E. (1987) *J. Biol. Chem. 262*, 15037-15087.
Rosen, G., Gresser, M., Vinkler, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 10654.
Satre, M., Bof, M., & Vignais, P. V. (1980) *J. Bacteriol. 134*, 17-23.
Schumann, J., Richter, M. L., & McCarty, R. E. (1985) *J. Biol. Chem. 260*, 11817-11823.
Senior, A. E. (1988) *Physiol. Rev. 68*, 177-231.
Sternweis, P. C., & Smith, J. B. (1977) *Biochemistry 16*, 4020-4025.
Sternweis, P. C., & Smith, J. B. (1980) *Biochemistry 19*, 526-531.
Towbin, H., Staehlin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Wood, J. M., Wise, J. G., Senior, A. E., Futai, M., & Boyer, P. D. (1987) *J. Biol. Chem. 262*, 2180-2186.

Evidence from Total Internal Reflection Fluorescence Microscopy for Calcium-Independent Binding of Prothrombin to Negatively Charged Planar Phospholipid Membranes†

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ABSTRACT: Measurements to test for a proposed Ca^{2+}-independent interaction of prothrombin with membranes containing acidic phospholipids are described. Fluorescein-labeled bovine prothrombin and its amino- and carboxy-terminal peptides, prothrombin fragment 1 and prethrombin 1, were added at various concentrations in the presence or absence of Ca^{2+} to the aqueous space bathing substrate-supported planar membranes composed of 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), POPC/bovine brain phosphatidylserine (bovPS) (70:30 mol/mol), or POPC/1,2-dioleoyl-3-*sn*-phosphatidylglycerol (DOPG) (70:30 mol/mol). Total internal reflection fluorescence microscopy (TIRFM) at the membrane–solution interface showed a significant enhancement by acidic lipids of prothrombin and prothrombin fragment 1 binding in the presence of 5 mM Ca^{2+}, with apparent dissociation constants of 0.4 and 1 μM, respectively. TIRFM measurements indicated that bovPS and DOPG also significantly enhanced the binding of fluorescein-labeled prothrombin to the planar membranes in the absence of Ca^{2+}, with apparent dissociation constants (13–30 μM) at least an order of magnitude larger than the Ca^{2+}-dependent constant for prothrombin binding. Association of prethrombin 1 but not prothrombin fragment 1 with membranes in the absence of Ca^{2+} was enhanced by the presence of bovPS in the membranes, which suggests that the Ca^{2+}-independent binding site(s) is (are) in the prethrombin 1 but not the fragment 1 portion of prothrombin.

In the coagulation cascade, prothrombin is the substrate of the prothrombinase complex which consists of the enzyme factor X_a, its cofactor, factor V_a, and a platelet membrane or a negatively charged phospholipid vesicle. Factors X_a and V_a assemble on these phospholipid surfaces and accelerate the conversion of prothrombin to thrombin. The thrombin proteolysis products of prothrombin are prethrombin 1 (the C-terminal two-thirds of the prothrombin molecule) and fragment 1 (the N-terminal third). The fragment 1 portion of prothrombin contains doubly negatively charged γ-carboxyglutamic acid residues. Binding of prothrombin in the presence of Ca^{2+} to negatively charged membranes is well documented and is reported to require the occupation of Ca^{2+}-binding sites in the fragment 1 portion and to involve the γ-carboxyglutamic acid residues (Suttie & Jackson, 1977; Jackson & Nemerson, 1980; Nemerson & Furie, 1980; Nelsestuen, 1984).

We have previously obtained evidence which suggests that bovine prothrombin interacts with phosphatidylserine (PS)[1]

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[1] Abbreviations: PS, bovPS, phosphatidylserine, bovine brain PS; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DOPG, 1,2-dioleoyl-3-*sn*-phosphatidylglycerol; NBD-PE, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylethanolamine; FITC, fluorescein-5-isothiocyanate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; TIRFM, total internal reflection fluorescence microscopy; K_d, equilibrium dissociation constant.

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containing membranes even in the absence of calcium (Tendian & Lentz, 1990). The Ca^{2+}-independent interaction appeared not to occur between prothrombin fragment 1 and PS-containing membranes, and thus the interaction site might be in the prethrombin 1 region of prothrombin which contains the peptide bonds that are cleaved during thrombin formation. It has also been shown recently that binding to PS-containing membranes but not to phosphatidylglycerol (PG) containing membranes induced structural changes in the prethrombin 1 portion of prothrombin (Lentz et al., 1991; Wu & Lentz, 1991). Prothrombin is also known to be preferentially activated to thrombin on PS- as opposed to PG-containing membranes (Jones et al., 1985) apparently revealing a cofactor-like preference for PS as compared to other acidic phospholipids. It may be, then, that the prethrombin 1 region of prothrombin contains a binding site specific for PS, the occupancy of which triggers a functionally important conformational change. These considerations make the demonstration and characterization of the possible Ca^{2+}-independent prothrombin–membrane interaction of importance.

Calcium-independent interactions of human prothrombin with monolayers (Lecompte et al., 1980; Lecompte & Miller, 1980; Lecompte & Dode, 1989) and of bovine or human prothrombin with vesicles (Nelsestuen et al., 1978; Lecompte et al., 1984; Prigent-Dachary et al., 1986, 1989; Tendian & Lentz, 1990) have been reported previously. However, the putative Ca^{2+}-independent interaction of prothrombin with PS-containing membranes is apparently weak, and weak interactions are difficult to demonstrate as distinct from artifacts. For example, nonspecific adsorption can obscure specific binding to both lipid monolayers and bilayers at low surface densities. Monolayer binding studies are also complicated by the fact that the adsorption of a protein to an air/water interface need not occur by the same mechanism involved in binding to a membrane surface. In addition, the Ca^{2+}-independent interaction has been characterized mainly by indirect measurements, such as changes in monolayer surface pressure or capacitance (Lecompte & Miller, 1980; Lecompte & Dode, 1989) or changes in membrane packing order in unilamellar vesicles (Tendian & Lentz, 1990). The most direct demonstrations of the Ca^{2+}-independent interaction (via fluorescence resonance energy transfer) have produced unreasonably large binding constants (Prigent-Dachary et al., 1986, 1989). The purposes of this report are to present direct measurements of the Ca^{2+}-independent interaction with membrane bilayers and to address the possibility of nonspecific adsorption through the use of different membrane compositions and different proteolytic fragments of prothrombin.

Total internal reflection fluorescence microscopy (TIRFM)[1] (Axelrod et al., 1984) was used to directly detect the association of fluorescently labeled bovine prothrombin and its proteolytic fragments with phospholipid membranes deposited on planar solid substrates (McConnell et al., 1986; Thompson et al., 1988). In TIRFM, a laser beam is made incident at an oblique angle on the dielectric interface of a solution containing fluorescently labeled ligand and a transparent planar substrate. The internal reflection of the laser beam creates a thin layer of illumination adjacent to the surface called the evanescent field, which decays exponentially into the solution. Only those molecules adsorbed to the surface or in the solution within a small distance from the surface are excited by the evanescent field and subsequently fluoresce. Membrane equilibrium dissociation constants (K_d)[1] at surfaces coated with model membranes may be determined from TIRFM measurements of the fluorescence as a function of the concentration of fluorescently labeled ligand in solution. TIRFM has recently been used to examine the association of a variety of biochemical ligands with different surfaces (Darst et al., 1988; Sui et al., 1988; Hlady et al., 1988, 1989; Fraaije et al., 1990; Kalb et al., 1990; Pisarchick & Thompson, 1990; Poglitsch & Thompson, 1990; Schmidt et al., 1990). In the work described herein, we report evidence in support of a Ca^{2+}-independent, acidic lipid-dependent prothrombin–membrane interaction involving the prethrombin 1 domain of bovine prothrombin but not the fragment 1 domain.

Experimental Procedures

Protein Purification. Prothrombin was purified from bovine plasma by barium citrate precipitation, ammonium sulfate fractionation, anion exchange chromatography, and Bio-Rad P-100 gel filtration chromatography (Tendian & Lentz, 1990). Prothrombin which had been passed over burro anti-bovine factor V–Sepharose to remove traces of factor V (Boskovic et al., 1990) was obtained for a control experiment through the generosity of Willem Stevens and Michael Nesheim of Queen's University. Prothrombin fragment 1 and prethrombin 1 were isolated as the thrombin proteolysis products of prothrombin. Prothrombin in 20 mM Tris, 100 mM NaCl, and 0.02% NaN_3, pH 7.4, was digested for 3 h at 37 °C by one unit of bovine thrombin (600 NIH units/mg) (Sigma Chemical Co., St. Louis, MO) per milligram of bovine prothrombin (Heldebrant et al., 1973). Prethrombin 1 and fragment 1 from the digestion mixture were isolated by anion exchange chromatography on DEAE-cellulose or on a Perkin-Elmer Isopure LC system using a Pharmacia (Norwalk, CT) MonoQ HR 5/5 column. Fragment 1 was further purified by Bio-Rad P-100 gel filtration.

Proteins were judged to be >95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentrations were determined by absorbance measurements using absorptivities at 280 nm of 1.44 mL mg^{-1} cm^{-1}, 1.64 mL mg^{-1} cm^{-1}, and 1.05 mL mg^{-1} cm^{-1} for prothrombin, prethrombin 1, and fragment 1, respectively (Mann, 1976). The 280 nm absorbance was corrected for light scattering using the 320 nm absorbance (Donovan, 1969). Prothrombin concentrations were confirmed by using the synthetic chromogenic substrate S2238 (KabiVitrum, Stockholm, Sweden) to assay thrombin formation from prothrombin activated by *Echis carinatus* snake venom (Sigma Chemical Co., St. Louis, MO) (Rosing et al., 1980). The intrinsic fluorescence of purified fragment 1 was quenched by 41–51% (λ_{ex} = 295 nm, λ_{em} = 345 nm) after addition of 5 mM $CaCl_2$ (Nelsestuen, 1976; Prendergast & Mann, 1977; Nelsestuen et al., 1981).

Fluorescence Labeling. Purified prothrombin, fragment 1, and prethrombin 1 in 50 mM sodium bicarbonate buffer, pH 9.2, and at 0.7–3.2 mg/mL were labeled with fluorescein-5-isothiocyanate (FITC,[1] Molecular Probes, Eugene, OR) by adding a 75-fold (prothrombin and prethrombin 1) or 150-fold (fragment 1) molar excess of FITC in *N,N*-dimethylformamide so that the volume of added FITC solution was <5% of the protein solution volume. The protein was incubated 1 h in the dark at room temperature with periodic mixing. Free dye was separated from labeled protein by gel filtration using Sephadex G-25 (Pharmacia-LKB, Piscataway, NJ). Observation of SDS–PAGE gels under ultraviolet illumination indicated that the proteins were fluorescently labeled and that no detectable free FITC was present. The concentrations of labeled protein and the molar ratios of fluorescein to protein were determined spectrophotometrically assuming molar absorptivities of bound FITC equal to 72 mM^{-1} cm^{-1} at 494 nm and 26 mM^{-1} cm^{-1}

at 280 nm (Goldman, 1968; Mishell & Shiigi, 1980). The FITC/protein molar ratios ranged from 0.8–1.6 for prothrombin, 1.2–1.3 for prethrombin 1, and 0.3–0.5 for fragment 1.

For storage, labeled protein solutions were brought to 10 mM Na_2EDTA,[1] dialyzed into dilute TES[1] buffer (~0.1 mM TES, 1 mM NaCl, and 0.0002% NaN_3), lyophilized, and frozen at −20 °C. Immediately prior to use, lyophilized protein was dissolved in TES buffer (10 mM TES, 100 mM NaCl, 0.02% NaN_3, pH 7.4, with 5 mM $CaCl_2$ or with 1 mM Na_2EDTA). The buffer containing EDTA was passed over Chelex-100 (Bio-Rad) to remove divalent cations. Prethrombin 1 and fragment 1 were used within 2 months of labeling and prothrombin was used within 2 weeks of labeling. Protein solutions were not thawed for longer than 48 h. Labeled fragment 1 showed approximately the same fluorescence quench (41–49%) (λ_{ex} = 295 nm, λ_{em} = 345 nm) as unlabeled fragment 1 after addition of 5 mM $CaCl_2$.

Phospholipid Vesicles. Small unilamellar vesicles were prepared from phospholipid/chloroform stock solutions (Avanti Polar Lipids, Birmingham, AL). After chloroform evaporation, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC),[1] POPC/bovine brain phosphatidylserine (bovPS)[1] mixtures (70:30 mol/mol), and POPC/1,2-dioleoyl-3-*sn*-phosphatidylglycerol (DOPG)[1] mixtures (70:30 mol/mol) were lyophilized from frozen benzene or cyclohexane solutions, hydrated to a concentration of 2 mM with TES buffer, and sonified in a Heat Systems W350 Sonicator (Lentz et al., 1982). 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]dodecanoyl]phosphatidylethanolamine (NBD-PE,[1] Avanti Polar Lipids) was incorporated at 2 mol % prior to lyophilization in vesicles used for lateral diffusion measurements. Vesicles were maintained above their phase transition temperature after hydration and fractionated by ultacentrifugation in a Beckman TL-100 centrifuge for 25 min at 200000*g* immediately before use.

Substrate-Supported Planar Membranes. Substrate-supported planar model membranes were formed using a procedure similar to previously published methods (Brian & McConnell, 1984; Poglitsch & Thompson, 1990). Immediately before use, detergent-cleaned fused silica substrates and glass microscope slides were treated with an argon ion plasma cleaner for 15 min (PDC-3XG, Harrick Scientific Corp., Ossining, NY), mounted together with a spacer of thickness ≃100 μm, treated with 75 μL of sonicated vesicles for 20 min, and washed with 3 mL of TES buffer with or without Na_2EDTA.

Lateral Diffusion Measurements. The lateral mobility of NBD-PE incorporated in supported membranes was measured with fluorescence pattern photobleaching recovery (Smith & McConnell, 1978). The radii of the illuminated and observed circular areas in the sample plane, formed with a beam expander (5×, Oriel Corp., Stratford, CT) and an image plane aperture, respectively, were >225 and 75 μm. Other parameters were as previously described (Wright et al., 1988) except as follows: laser wavelength, 488.0 nm; observation and bleaching laser powers, 0.075–1.5 μW and 0.3–0.6 W; bleach pulse duration, 100–750 ms; ruling periodicity, 16 μm. For each of the three membrane compositions, measurements were made on at least two different vesicle preparations. Best-fit values of diffusion coefficients and fractional mobilities were obtained using known theoretical forms and a nonlinear curve-fitting routine (Wright et al., 1988; Timbs et al., 1991).

Total Internal Reflection Fluorescence Microscopy (TIRFM). TIRFM (Axelrod et al., 1984; Thompson et al., 1988) was used to monitor the association of labeled proteins with substrate-supported planar membranes. Planar membranes were first treated for 10 min with 250 μL of 5 mg/mL unlabeled sheep immunoglobulin (Sigma Chemical Co., St. Louis, MO) that had been clarified at ~200000*g* for 25–30 min to block nonspecific binding sites that may have been present on the membranes. Membranes were then treated with a 240-μL volume containing 5 mg/mL sheep immunoglobulin and various concentrations of fluorescein-labeled prothrombin, fragment 1, or prethrombin 1 in TES buffer containing 1 mM Na_2EDTA or 5 mM Ca^{2+}. The fluorescence intensities of proteins excited by the evanescent field were measured with a fluorescence microscope equipped for TIRFM (Poglitsch & Thompson, 1990; Pisarchick & Thompson, 1990). Parameters were as previously described except as follows: laser wavelength and power, 488 nm and ~500 nW; incidence angle, 75°; illuminated area, 20 μm × 200 μm; refractive indices, 1.467 for fused silica and 1.334 for water; and evanescent field depth, ~800 Å. If data for one of the binding curves came from different vesicle and protein preparations, then the fluorescence intensities were normalized by comparing measurements made with different preparations at the same protein concentration(s). Experimentally obtained equilibrium binding curves were fit to theoretical forms using the SIMPLEX nonlinear least squares curve-fitting routine (Caceci & Cacheris, 1984).

Emission Spectra of Fluorescein-Labeled Protein. Emission spectra (λ_{ex} = 488 nm, λ_{em} = 505–600 nm) of fluorescein-labeled prothrombin (4.2×10^{-8} M), fragment 1 (1.3×10^{-7} M), and prethrombin 1 (4.2×10^{-8} M) in TES buffer were measured with an SLM 48000 spectrofluorometer (SLM Aminco, Urbana, IL) in the presence and absence of 15 mM bovPS/POPC (30:70 mol/mol) or POPC small unilamellar vesicles with 1 mM Na_2EDTA or 5 mM $CaCl_2$. Spectra of fluorescein-labeled prothrombin were also measured in the presence of DOPG/POPC (30:70 mol/mol) small unilamellar vesicles. The intensities of the background-corrected emission maxima were compared to determine if association of protein with vesicles of different compositions changed the fluorescence quantum yield.

Table I: Lateral Diffusion of NBD-PE in Substrate-Supported Membranes[a]

membrane comp	diffusion coeff (10^{-8} cm^2/s)	fractional mobility
98:2 POPC/NBD-PE	1.6 ± 0.1	0.59 ± 0.01
30:68:2 bovPS/POPC/NBD-PE	1.5 ± 0.3	0.65 ± 0.03
30:68:2 DOPG/POPC/NBD-PE	1.2 ± 0.1	0.69 ± 0.03

[a] Membranes were formed on fused silica substrates. Values are averages for 2–5 membranes formed from two different vesicle preparations. Uncertainties are standard errors in the mean.

Results

Substrate-Supported Planar Membranes. Planar membranes containing NBD-PE showed some bright features (0.5–5 μm dots and occasional 30-μm long linear regions), but the majority of the fluorescence was uniformly distributed over the surface (within optical resolution). The apparent lateral diffusion coefficients and fractional mobilities of NBD-PE incorporated in POPC, bovPS/POPC, and DOPG/POPC supported membranes were consistent with formation of relatively uniform continuous membranes with laterally mobile lipids (Table I). The average apparent diffusion coefficient and fractional mobility of NBD-PE in the three types of membranes on fused silica substrates were ~10^{-8} cm^2/s and ~64%, respectively. The lateral diffusion coefficients were

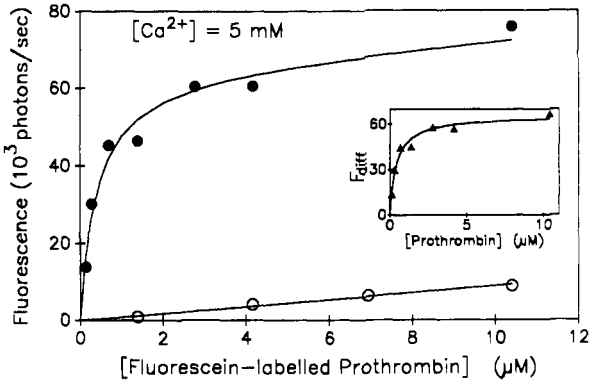

FIGURE 1: TIRFM of prothrombin bound to supported planar membranes in the presence of Ca^{2+}. A linear fit of the fluorescence of labeled prothrombin versus applied prothrombin concentration measured by TIRFM on planar POPC membranes (open circles) was subtracted from the fluorescence measured on planar bovPS/POPC membranes (closed circles). The differences are shown in the inset (triangles). The best fit of the fluorescence differences to eq 1 (inset) was added to the linear fit of the fluorescence on POPC membranes to obtain the fit shown for the bovPS/POPC data. Data points were single measurements obtained with one vesicle preparation and one prothrombin preparation.

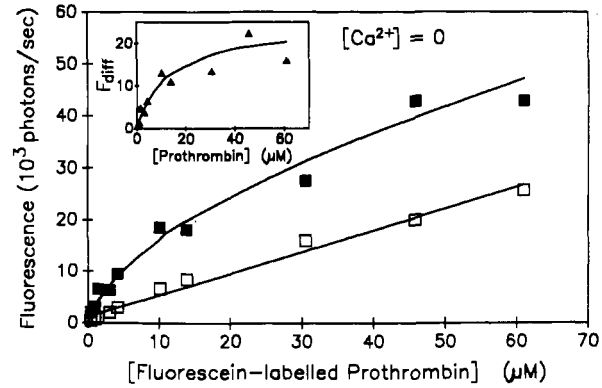

FIGURE 2: TIRFM of prothrombin bound to supported planar membranes in the absence of Ca^{2+}. The fluorescence of labeled prothrombin on planar bovPS/POPC (closed squares) and POPC (open squares) membranes in the absence of Ca^{2+} is plotted and fit as in Figure 1 except for the exclusion of the highest prothrombin concentration point when fitting the difference data. Data were obtained with three different vesicle preparations and four different prothrombin preparations. Approximately half of the points are averages of duplicate measurements.

similar to those measured for cellular and vesicular membrane lipids (Cherry, 1976; Peters, 1981; Jacobson, 1983). Glass substrates were also tested as supports for planar membranes; in these samples, the NBD-PE lateral diffusion coefficients were ~1.5 fold higher and the fractional mobilities were ~1.2 fold higher than the diffusion coefficients and fractional mobilities on fused silica supports.

Interaction of Prothrombin with Planar Membranes. The relative fluorescence intensities of fluorescein-labeled proteins excited by the evanescent waves created by a laser beam totally internally reflected at the supported planar membrane/solution interfaces are shown in Figures 1–5 as a function of the total concentrations of protein added to the aqueous spaces contacting acidic or neutral membranes.

Measurements of bovine prothrombin binding to membranes in the presence of Ca^{2+} are shown in Figure 1. The fluorescence on bovPS/POPC membranes (closed circles) showed an apparently saturating increase in intensity with increasing prothrombin concentration, whereas the fluorescence on POPC membranes (open circles) was greatly decreased for the same prothrombin concentration range. The small linear increase in fluorescence on POPC membranes may be attributed at least in part to solution fluorescence arising from the finite evanescent field depth.

To eliminate the solution fluorescence contributions in the quantitation of the Ca^{2+}-dependent PS-specific binding of prothrombin, the fluorescence calculated from a linear fit of the fluorescence on POPC membranes was subtracted from the fluorescence measured on bovPS/POPC membranes. The fluorescence differences, F_{diff} were fit to

$$F_{diff} = \frac{F_\infty[P]}{K_d + [P]} \quad (1)$$

where [P] is the protein solution concentration and was approximated as the known bulk concentration of protein added to the aqueous space bathing the membranes, and the equilibrium dissociation constant K_d and the fluorescence difference at saturation F_∞ were free parameters. This analysis yielded K_d = 0.4 μM for the Ca^{2+}-dependent association of prothrombin with PS-containing membranes.

In the absence of Ca^{2+}, the fluorescence from labeled prothrombin was also higher on bovPS/POPC membranes (closed squares) than on POPC membranes (open squares) (Figure 2). The fluorescence on bovPS/POPC membranes was ~5-fold less intense without Ca^{2+} than with Ca^{2+} between 2 and 10 μM prothrombin. The fluorescence on POPC membranes was approximately equal in the presence and absence of Ca^{2+} in the prothrombin concentration range where both types of data were obtained (≤10 μM). Higher protein concentrations were needed to measure the weaker Ca^{2+}-independent interaction with bovPS/POPC membranes, and consequently the data farther out on the curves in Figure 2 contain a larger intensity contribution due to solution fluorescence and possibly to nonspecific adsorption. To obtain an estimate of the K_d for the PS-specific Ca^{2+}-independent binding, the POPC data were subtracted from the bovPS/POPC data. The highest concentration point was excluded when fitting the difference data to eq 1; this analysis yielded K_d = 13 μM. The best fit of the fluorescence intensities on bovPS/POPC membranes (without subtracting the POPC data or excluding the final point) to eq 1 gave K_d = 30 μM. The justification for these two methods of analysis is given under Discussion.

Complete binding isotherms were not obtained with PG-containing membranes, but Table II shows the fluorescence intensities measured for two prothrombin concentrations in contact with DOPG/POPC membranes in the presence and absence of Ca^{2+}. Measurements with bovPS/POPC and POPC membranes are also given for comparison. For both prothrombin concentrations in the presence of Ca^{2+}, the fluorescence intensities increased from POPC, to DOPG/POPC, to bovPS/POPC membranes. The lower fluorescence intensities on DOPG/POPC membranes than on bovPS/POPC membranes probably reflected slightly weaker Ca^{2+}-dependent binding to PG/PC membranes consistent with previous reports (Nelsestuen & Broderius, 1977; Wei et al., 1982; Cutsforth et al., 1989). Without Ca^{2+}, both prothrombin concentrations showed approximately equivalent intensities on DOPG/POPC and bovPS/POPC membranes but lower intensities on POPC membranes.

Interaction of Prethrombin 1 with Planar Membranes. Similar to prothrombin, prethrombin 1, the carboxy-terminal peptide of prothrombin, showed a preference for bovPS/POPC membranes (closed squares) over POPC membanes (open squares) in the absence of Ca^{2+} (Figure 3). The fluorescence intensities with POPC membranes in the absence of Ca^{2+} were approximately equal for prothrombin and prethrombin 1

Table II: Comparison of Prothrombin Binding to Membranes of Different Compositions[a]

membrane comp	[prothrombin] = 1.4 μM rel fluorescence	[prothrombin] = 10 μM rel fluorescence
	Ca^{2+}	Ca^{2+}
bovPS/POPC	0.65 (0.61–0.69)	1.0
DOPG/POPC	0.32 (0.30–0.34)	0.59 ± 0.05
POPC	0.014 (0.013–0.016)	0.12 (0.116–0.121)
	EDTA[b]	EDTA[b]
bovPS/POPC	0.09 (0.07–0.10)	0.20 ± 0.08
DOPG/POPC	0.07 ± 0.01	0.18 ± 0.06
POPC	0.013 (0.011–0.014)	0.095 ± 0.003

[a] Bovine prothrombin was fluorescein labeled. [Ca^{2+}] = 5 mM. [Na_2EDTA] = 1 mM. BovPS/POPC and DOPG/POPC substrate-supported planar membranes contained 30 mol % acidic phospholipid. All fluorescence intensities were normalized to the fluorescence of 10 μM prothrombin with Ca^{2+} on bovPS/POPC membranes. The mean and standard deviation of measurements on four or more membranes are given for some of the data. For the rest of the data, measurements were made on fewer membranes so the mean and range are given. [b] The mean fluorescence intensities on bovPS/POPC and DOPG/POPC membranes with EDTA were significantly higher than on POPC membranes as determined by a t test (p = 0.01 and 0.025 for the bovPS/POPC and POPC comparison, and p = 0.0005 and 0.01 for the DOPG/POPC and POPC comparison with 1.4 and 10 μM prothrombin, respectively).

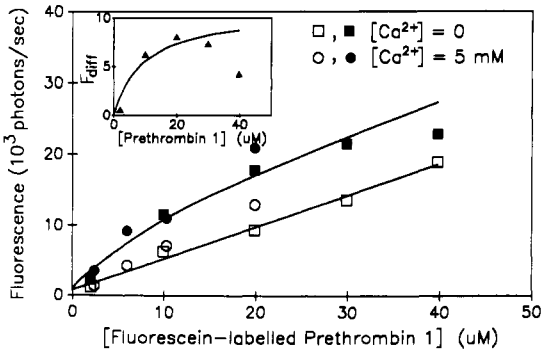

FIGURE 3: TIRFM of prethrombin 1 bound to supported planar membranes. Fluorescein-labeled prethrombin 1 was added to planar bovPS/POPC (closed symbols) and POPC (open symbols) membranes in the absence of Ca^{2+} (squares) or in the presence of Ca^{2+} (circles). Data in the absence of Ca^{2+} are averages of duplicate or triplicate measurements obtained with two different vesicle preparations and one prethrombin 1 preparation. A few single measurements with Ca^{2+} are included for comparison. The data analysis was for the data in the absence of Ca^{2+} and was identical to the analysis used for prothrombin in Figure 2. The fluorescein to protein molar ratios of prothrombin and prethrombin 1 were similar, ~1.5 and ~1.3, respectively, allowing direct comparison of fluorescence intensities in Figures 2 and 3.

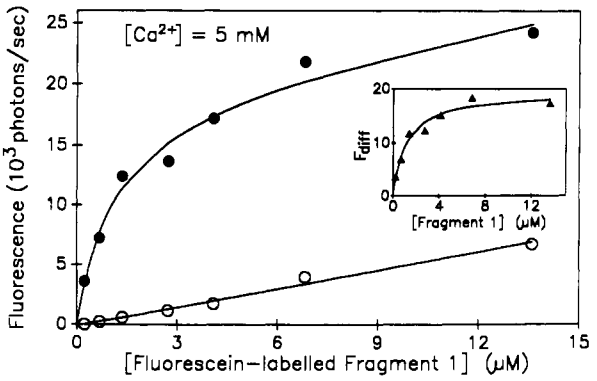

FIGURE 4: TIRFM of fragment 1 bound to supported planar membranes in the presence of Ca^{2+}. The fluorescence intensities of labeled fragment 1 on planar bovPS/POPC (closed circles) and POPC (open circles) membranes were fit with the procedure described in Figure 1. Due to the lower probe to protein molar ratio of labeled fragment 1 (~0.5) relative to prothrombin (~1.3), the fluorescence in Figures 1 and 4 can only be compared qualitatively. Data points were single measurements obtained with two different vesicle preparations and one fragment 1 preparation.

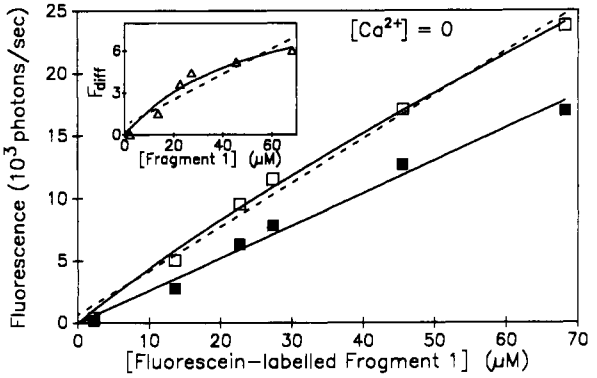

FIGURE 5: TIRFM of fragment 1 bound to supported planar membranes in the absence of Ca^{2+}. The fluorescence was higher on POPC membranes (open squares) than on bovPS/POPC membranes (closed squares) so, unlike in Figures 1–4, the fluorescence difference data (open triangles in inset) were calculated from the linear fit of the fluorescence measured on bovPS/POPC membranes (closed squares) subtracted from the fluorescence measured on POPC membranes (open squares). The difference data were fit both to eq 1 (solid curve) and to a straight line (dashed line) and were added to the linear fit of the bovPS/POPC data to obtain the fits of the POPC data. Data were obtained with three different vesicle preparations and two different fragment 1 preparations. Approximately half of the points are averages of duplicate or triplicate measurements. Due to the lower probe to protein ratio of labeled fragment 1 (~0.4) relative to prothrombin (~1.5) and prethrombin 1 (~1.3), the fluorescence in Figures 2, 3, and 5 can only be compared qualitatively.

(compare open squares in Figures 2 and 3), but the intensities with bovPS/POPC membranes were somewhat lower for prethrombin 1 than for prothrombin. The K_d obtained from fitting the difference data to eq 1, excluding the highest concentration data point, was 9 μM, whereas the best fit of the bovPS/POPC data to eq 1, without excluding the last data point, was obtained with K_d = 17 μM. Since the focus of our study was Ca^{2+}-independent binding, the fluorescence of prethrombin 1 in the presence of Ca^{2+} was not measured extensively; however, a few measurements are included in Figure 3 (circles). The fluorescence intensities for prethrombin 1 on bovPS/POPC membranes were approximately equal in the presence and absence of Ca^{2+}, indicating that prethrombin 1 did not exhibit Ca^{2+}-dependent binding.

Interaction of Fragment 1 with Planar Membranes. Shown in Figure 4 is the fluorescence of fluorescein-labeled prothrombin fragment 1 in the presence of Ca^{2+} on bovPS/POPC (closed circles) and POPC membranes (open circles). As with the parent prothrombin, the binding of fragment 1 to bovPS/POPC membranes was much greater than the binding to pure POPC membranes. The dissociation constant obtained from the best fit of the fluorescence difference data to eq 1 was K_d = 1 μM.

Unlike for prothrombin and prethrombin 1, the fluorescence intensity of labeled fragment 1 in the absence of Ca^{2+} was higher on POPC membranes (open squares) than on bovPS/POPC membranes (closed squares) (Figure 5). The fluorescence from fragment 1 with POPC membranes was similar whether or not Ca^{2+} was present (open symbols in Figures 4 and 5) but was higher than with bovPS/POPC membranes in the absence of Ca^{2+} (closed squares Figure 5). It is difficult to say from these data that preferential binding was occurring to POPC membranes in the absence of Ca^{2+}, since both the bovPS/POPC and POPC data were adequately

described by linear equations (dashed line). However, if we were to assume a preferential binding model for these data, the fluorescence differences (open triangles in inset) could be calculated from the linear fit of the fluorescence on bovPS/POPC membranes subtracted from the intensities on POPC membranes. The K_d determined in this way from the difference data and eq 1 was ≥ 52 μM. The apparent binding was not close to saturation, however, since both the bovPS/POPC and the POPC intensity data were adequately described by linear equations, and therefore the actual K_d may be much larger.

Interaction of Prothrombin from Anti-(Factor V) Column with Planar Membranes. Fluorescence intensities for one preparation of fluorescein-labeled prothrombin that had been passed down an anti-bovine factor V–Sepharose column were measured at 10 μM prothrombin with POPC, bovPS/POPC, and DOPG/POPC planar membranes in the absence of Ca^{2+} and with bovPS/POPC membranes in the presence of Ca^{2+}. Because limited quantities of material were available for these controls, measurements were made at single concentrations of prothrombin instead of over a range of concentrations to produce titration curves. As with prothrombin that was not exposed to polyclonal anti-bovine factor V, the fluorescence intensities on bovPS/POPC and DOPG/POPC membranes in the absence of Ca^{2+} were approximately equivalent, higher than the intensities on POPC membranes, and much lower than the intensities on bovPS/POPC membranes in the presence of Ca^{2+} (data not shown).

Interaction of Free FITC with Planar Membranes. FITC solutions were added to substrate-supported planar membranes to determine if unbound fluorescein could bind preferentially to bovPS/POPC membranes and thereby explain the higher fluorescence observed for prothrombin and prethrombin 1 on bovPS/POPC membranes. At a FITC concentration equivalent to the concentration of fluorescein bound to 30 μM prothrombin at a probe to protein molar ratio of 1.4, the fluorescence was 1.8–3.0 times higher on POPC than on bovPS/POPC supported planar membranes (data not shown).

Spectral Properties of Labeled Proteins. The fluorescence emission spectra of fluorescein-labeled prothrombin, prethrombin 1, and fragment 1 in the presence of Ca^{2+} or EDTA were measured in a cuvette in the presence and absence of membrane vesicles. The membrane concentration used for measurements with and without Ca^{2+} was sufficient to bind >50% of the prothrombin to bovPS/POPC vesicles even in the absence of Ca^{2+}. The ratios of the fluorescence intensities of the three proteins with POPC vesicles relative to the intensities measured with bovPS/POPC vesicles ranged from $\sim$1.1 to 1.4 both in the presence and absence of Ca^{2+} (data not shown). The ratios of the fluorescence intensities of prothrombin with POPC vesicles relative to DOPG/POPC vesicles were $\sim$1.1 both in the presence and absence of Ca^{2+} (data not shown).

Discussion

Properties of Supported Membranes. Substrate-supported planar membranes have been formed by depositing sonicated small unilamellar vesicles containing neutral lipids or mixed neutral and acidic lipids on fused silica and glass substrates. Similar substrate-supported membranes have been made previously from neutral synthetic vesicles (Brian & McConnell, 1984; Poglitsch & Thompson, 1990). Of interest is the result that acidic membranes will form on Ar^+-treated, negatively charged silicon dioxide surfaces. The long-range lipid lateral mobility supports the hypothesis that vesicle fusion can be catalyzed by solid surfaces. The bright features observed in membranes containing NBD-PE could have been vesicles which absorbed but did not fuse and could account for the observed incomplete mobility. The fact that lateral diffusion rates were comparable to rates in other artificial or natural membranes supported the legitimacy of using this technique to form model membranes. Fused silica rather than glass substrates were used for TIRFM because the background intensity was higher on glass substrates.

Binding of Prothrombin and Prothrombin Fragments to Supported Membranes. TIRFM was well-suited to measurements of the binding of prothrombin and prothrombin fragment 1 in the *presence of* Ca^{2+} where the K_d's were in the micromolar and submicromolar range. Throughout the titrations, the intensities measured on bovPS/POPC and DOPG/POPC membranes were much larger than the intensities measured on POPC membranes, and the surface appeared to approach saturation well before the protein solution concentration (and possibly nonspecific adsorption) contributed significantly to the fluorescence excited by the evanescent wave. The acidic lipid dependence of the Ca^{2+}-dependent binding of prothrombin and prothrombin fragment 1 was therefore clearly demonstrated by these measurements. The prothrombin–Ca^{2+}–PS/PC interaction provided a comparison of TIRFM on planar membranes to 90° light-scattering measurements used previously to quantitate binding of bovine prothrombin to lipid vesicles (Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977). The dissociation constants for the PS-specific, Ca^{2+}-dependent binding of bovine prothrombin and fragment 1 (0.4 and 1 μM, respectively) to supported planar membranes agreed well with literature values for bovine prothrombin binding to PS/PC lipid vesicles [K_d = 0.3–0.4 μM (Nelsestuen & Broderius, 1977; Resnick & Nelsestuen, 1980; Wei et al., 1982)] and reasonably well for bovine prothrombin fragment 1 [$K_d \approx 1$ μM (Dombrose et al., 1979) and K_d = 0.3 μM (S. Tendian and B. Lentz, unpublished data)]. The similar K_d's for labeled and unlabeled proteins indicate that the affinity of FITC-labeled prothrombin and prothrombin fragment 1 for bovPS/POPC membranes was not dramatically affected by the labeling procedure.

The data in the *absence of* Ca^{2+} also pointed to an acidic lipid dependence of prothrombin binding, although the data were less quantitatively precise than in the presence of Ca^{2+}. The Ca^{2+}-independent association was equivalent on the two negatively charged membranes (Table II), indicating that there was not a requirement for a particular acidic phospholipid. At higher protein concentrations, it is likely that the solution fluorescence contributed significantly to the total fluorescence. In addition to high solution fluorescence, at least part of the significant fluorescence measured on POPC membranes at higher prothrombin concentrations may have been due to nonspecific surface binding of prothrombin. FITC-labeled insulin exhibits similar behavior by binding somewhat to DMPC supported bilayers but binding more strongly to bilayers containing negatively charged DMPS or ganglioside (Sui et al., 1988).

Prethrombin 1 showed a similar preference for bovPS/POPC membranes in the absence of Ca^{2+}. The fluorescence observed on POPC membranes was comparable for measurements with prothrombin and prethrombin 1, indicating that the nonspecific absorption of prethrombin 1 was not greater than that of prothrombin even though prethrombin 1 is a protein fragment. However, although the K_d of prethrombin 1 binding to bovPS/POPC membranes was approximately equivalent to that of prothrombin, the fluorescence intensities, normalized by the molar ratios of probe to protein,

were smaller by a factor of 1.2–1.4, perhaps suggesting a decrease in the density of surface binding sites.

Control measurements showed that the higher fluorescence of fluorescein-labeled prothrombin and prethrombin 1 on bovPS/POPC or DOPG/POPC membranes was not due to a larger quantum yield of these proteins when bound to bovPS/POPC or DOPG/POPC membranes relative to the yield when bound to POPC membranes. The fluorescence difference also could not be explained by preferential binding of free FITC, because free FITC adsorbed preferentially to POPC rather than to bovPS/POPC membranes. The observation that the fluorescence due to prothrombin fragment 1 was higher for POPC membranes than for bovPS/POPC membranes in the absence of Ca^{2+} indicated that the prothrombin fragment 1 domain was not responsible for the Ca^{2+}-independent, PS-dependent binding of prothrombin nor was the PS preference due to enhanced nonspecific protein adsorption to bovPS/POPC versus POPC planar membranes. The higher fluorescence of fragment 1 measured with POPC membranes in the absence of Ca^{2+} could indicate a very weak binding preference of fragment 1 for POPC membranes.

Estimation of Binding Constants and Binding Site Densities. The range of binding constants which can be measured by TIRFM is theoretically limited by the requirement that a significant fraction of the total fluorescence must originate from bound molecules. The upper limit for measurable dissociation constants is approximately equal to the product of the evanescent wave depth and the surface site density (Thompson et al., 1988). The ratio r of the fluorescence on PS/PC and PC membranes is, in the absence of adsorption to PC membranes

$$r = \frac{[S] + d[P]}{d[P]} \quad (2)$$

where [P] is the prothrombin solution concentration, d is the evanescent field depth (~800 Å), and [S] is the surface density of bound prothrombin. The value of [S] calculated from eq 2 is a lower bound for the surface density which does not account for possible nonspecifically adsorbed protein.

The surface site densities [S] at saturation on bovPS/POPC supported membranes estimated from eq 2 range from ≥3500 prothrombin molecules/μm^2 [≤440 lipid molecules/prothrombin molecule assuming a lipid surface area of 65 $Å^2$ (Cornell & Separovic, 1983; De Young & Dill, 1988)] in the presence of Ca^{2+} to ≥1150 prethrombin 1 molecules/μm^2 (≤1340 lipid molecules/prethrombin 1 molecule) in the absence of Ca^{2+}. These densities suggest much lower coverage on the supported membranes than on phospholipid vesicles, since the stoichiometry measured by light scattering for prothrombin bound to 20:80 PS/PC vesicles in the presence of Ca^{2+} was ~51 outer leaflet lipids per bound prothrombin (Cutsforth et al., 1989).

The low surface site densities observed for Ca^{2+}-independent binding make it difficult to measure dissociation constants for these weak binding interactions since, at high protein concentrations, the solution fluorescence and/or the fluorescence from nonspecifically adsorbed protein is equal to or greater than the fluorescence from bound molecules. It is difficult to know the extent to which these two effects contribute to the observed background and, thus, how to properly treat the background in analyzing the data. If the background were principally due to solution protein excited by the evanescent field, it would be appropriate to subtract it to obtain an estimate of K_d. If the background were due to adsorption to the PC membranes, this PS-independent interaction might be considered a legitimate contributor to the overall binding to bovPS/POPC membranes. In this case, subtraction of the background would be inappropriate. For these reasons, the dissociation constants for the Ca^{2+}-independent binding were calculated as ranges, with the lower limit determined from difference data and the upper limit determined from bovPS/POPC data.

Two additional effects could, in principle, complicate quantitative interpretation of TIRFM binding data. First, errors in dissociation constants could result from the assumption that the applied protein concentrations equaled the final protein solution concentrations. However, the amount by which bovPS/POPC membranes depleted the solutions of prothrombin, calculated using the Ca^{2+}-dependent surface site densities given above, was approximately 12%, 8%, and 4% at the first three points of the binding curve and was <1% for the highest prothrombin concentration. This amount of depletion would not significantly affect the calculated K_d. For the other binding curves, the depletion was much less, even for the lower concentration points. Second, at the high protein solution concentrations necessary to estimate the weak, Ca^{2+}-independent binding constants, significant fluctuations in the observed fluorescence might result from changes in the incidence angle which alter the depth of the exponentially decaying evanescent field and consequently modify the number of excited solution molecules. Indeed, large fluctuations made it very difficult to obtain accurate data at high protein concentrations. This limitation of the TIRFM technique requires that the reported K_d values for Ca^{2+}-independent binding be viewed as lower limit estimates to the actual K_d values.

A Hypothesis To Explain the Observed Ca^{2+}-Independent Prothrombin–Membrane Binding: Membrane Defects, Electrostatics, and a Specific Acidic Lipid-Binding Site. Protein impurities or membrane impurities (i.e., defects) might be suggested as a possible explanation for the measured membrane binding preferences for prothrombin and its proteolytic fragments, but the available data do not support this explanation. On the anion exchange column used for prothrombin purification and to separate fragment 1 and prethrombin 1, prothrombin and fragment 1 behave similarly but prothrombin and prethrombin 1 elute very differently. Therefore, a protein impurity would not likely copurify with prothrombin and prethrombin 1 and not copurify with fragment 1. Possible contamination by coagulation factor V, which binds to the fragment 2 region of prothrombin (Esmon & Jackson, 1974; Bajaj et al., 1975) via the heavy chain of factor V_a (Guinto & Esmon, 1984), was probably not responsible for the acidic lipid preference of the observed Ca^{2+}-independent membrane binding, since passage over anti-bovine factor V–Sepharose did not affect this aspect of the TIRFM results. Thus, the preference for acidic membranes exhibited by prothrombin and prethrombin 1 is probably a characteristic of these proteins and not due to a residual protein impurity.

The data suggest some binding of prothrombin and its peptides to all three of the membranes examined. Indeed, the drop in difference fluorescence data at high protein concentrations suggests that nonspecific absorption interferes with detection of specific interactions at high protein concentrations. Nonspecific absorption might be expected to reflect absorption to "defects" in the membranes (i.e., imperfections in the coating of the fused silica substrate sometimes observed as bright spots or lines on the substrates coated with NBD-PE and nonfluorescent lipid). For absorption to be truly nonspecific, roughly the same results should have been obtained for all the membranes and peptides considered, which was not observed.

Prothrombin and prethrombin 1 adsorbed preferentially to acidic lipid containing membranes, while fragment 1 preferred a neutral membrane containing only phosphatidylcholine. Considering general electrostatic effects only, it is not surprising that fragment 1, a highly acidic peptide, should absorb preferentially to a neutral as opposed to an acidic surface. However, prothrombin and prethrombin 1 are also acidic peptides, and either a specific interaction or possibly a hydrophobic interaction must be invoked to account for preferential binding of these peptides to acidic lipid membranes. One report has suggested that the Ca^{2+}-independent interaction between prothrombin and vesicles (Lecompte et al., 1984) might involve hydrophobic penetration of the bilayer by a portion of the prothrombin molecule. Our observation that prothrombin fragment 1 induces fusion of dimyristoylphosphatidylcholine (DMPC) small unilamellar vesicles (Lentz et al., 1985) argues for a perturbation of the hydrophobic membrane core by this protein fragment. This induction of fusion was unsaturable up to 40 μM fragment 1, suggesting a low-affinity interaction with neutral membranes, which may be due to exposure of hydrophobic residues. Prothrombin itself causes no fusion of either neutral or acidic lipid vesicles (Tendian & Lentz, 1990), arguing against the possibility of hydrophobic penetration by prothrombin. In addition, a hydrophobic interaction would not likely be specific for acidic lipid membranes as opposed to neutral lipid membranes. Altogether, the most likely explanation for the TIRFM measurements is that the prethrombin 1 region of prothrombin contains an acidic lipid dependent binding site. This hypothesis was substantiated by diphenylhexatriene anisotropy measurements which showed a prothrombin (but not fragment 1) induced decrease in the extent of acyl chain motion in DMPC/dimyristoylphosphatidylserine and DMPC/dipentadecanoylphosphatidylglycerol vesicles in both the presence and absence of Ca^{2+} (Tendian & Lentz, 1990).

Conclusions

Binding measurements using total internal reflection fluorescence microscopy confirmed that binding of fluorescein-labeled prothrombin in the absence of Ca^{2+} to bovPS/POPC or DOPG/POPC membranes was greater than to POPC membranes. Enhanced binding to bovPS/POPC membranes was also observed for carboxy-terminal prethrombin 1 but not for amino-terminal fragment 1, indicating that the site(s) of the Ca^{2+}-independent interaction is (are) in the prethrombin 1 domain of prothrombin. The dissociation constants estimated for the interaction of prothrombin and prethrombin 1 with bovPS/POPC substrate-supported planar membranes in the absence of Ca^{2+} were 13–30 and 9–17 μM, respectively. The combined use of TIRFM and planar membanes has provided a direct measurement of the Ca^{2+}-independent interaction with reasonable K_d estimates and has set the stage for future kinetic or steady-state measurements of membrane-associated coagulation factors.

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Registry No. POPC, 26853-31-6; DOPG, 62700-69-0; Ca, 7440-70-2; prothrombin, 9001-26-7; prothrombin fragment 1, 72270-84-9; prethrombin 1, 69866-47-3.

References

Axelrod, D., Burghardt, T. P., & Thompson, N. L. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 247–268.

Bajaj, S. P., Butkowski, R. J., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 2150–2156.

Boskovic, D. S., Giles, A. R., & Nesheim, M. E. (1990) *J. Biol. Chem. 265*, 10497–10505.

Brian, A. A., & McConnell, H. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6159–6163.

Caceci, M. S., & Cacheris, W. P. (1984) *Byte 8*, 340–361.

Cherry, R. J. (1976) in *Biological Membranes* (Chapman, D., & Wallach, D. F. H., Ed.) pp 47–102, Academic Press, New York.

Cornell, B. A., & Separovic, F. (1983) *Biochim. Biophys. Acta 733*, 189–193.

Cutsforth, G. A., Whitaker, R. N., Hermans, J., & Lentz, B. R. (1989) *Biochemistry 28*, 7453–7461.

Darst, S. A., Robertson, C. R., & Berzofsky, J. A. (1988) *Biophys. J. 53*, 533–539.

De Young, L. R., & Dill, K. A. (1988) *Biochemistry 27*, 5281–5289.

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

Donovan, J. W. (1969) in *Physical Principles and Techniques in Protein Chemistry Part A* (Leach, S. J., Ed.) pp 164–165, Academic Press, New York.

Esmon, C. T., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 7791–7797.

Fraaije, J. G. E. M., Kleijn, J. M., van der Graaf, M., & Dijt, J. C. (1990) *Biophys. J. 57*, 965–975.

Goldman, M. (1968) in *Fluorescent Antibody Methods*, pp 119–127, Academic Press, New York.

Guinto, E. R., & Esmon, C. T. (1984) *J. Biol. Chem. 259*, 13986–13992.

Heldebrant, C. M., Butkowski, R. J., Bajaj, S. P., & Mann, K. G. (1973) *J. Biol. Chem. 248*, 7149–7163.

Hlady, V., Rickel, J., & Andrade, J. D. (1988/1989) *Colloids Surf. 34*, 171–183.

Jackson, C. M., & Nemerson, Y. (1980) *Annu. Rev. Biochem. 49*, 765–811.

Jacobson, K. (1983) *Cell Motil. 3*, 367–373.

Jones, M. E., Lentz, B. R., Dombrose, F. A., & Sandberg, H. (1985) *Thromb. Res. 39*, 711–724.

Kalb, E., Engel, J., & Tamm, L. K. (1990) *Biochemistry 29*, 1607–1613.

Lecompte, M. F., & Miller, I. R. (1980) *Biochemistry 19*, 3439–3446.

Lecompte, M. F., & Dode, C. (1989) *Biochimie 71*, 175–182.

Lecompte, M. F., Miller, I. R., Elion, J., & Benarous, R. (1980) *Biochemistry 19*, 3434–3439.

Lecompte, M. F., Rosenberg, I., & Gitler, C. (1984) *Biochem. Biophys. Res. Commun. 125*, 381–386.

Lentz, B. R., Madden, S., & Alford, D. R. (1982) *Biochemistry 21*, 6799–6807.

Lentz, B. R., Alford, D. R., Jones, M. E., & Dombrose, F. A. (1985) *Biochemistry 14*, 6997–7005.

Lentz, B. R., Wu, J. R., Sorrentino, A. M., & Carleton, J. A. (1991) *Biophys. J.* (in press).

Mann, K. G. (1976) *Methods Enzymol. 45*, 123–156.

McConnell, H. M., Watts, T. H., Weis, R. M., & Brian, A. A. (1986) *Biochim. Biophys. Acta 864*, 95–106.

Mishell, B. B., & Shiigi, S. M. (1980) *Selected Methods in Cellular Immunology*, W. H. Freeman, San Francisco, CA.

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

Nelsestuen, G. L. (1984) in *Metal Ions in Biological Systems* (Sigel, H., Ed.) Vol. 17, pp 353–380, Marcel Dekker Inc., New York.
Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16*, 4172–4177.
Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164–4171.
Nelsestuen, G. L., Kisiel, W., & Di Scipio, R. G. (1978) *Biochemistry 17*, 2134–2138.
Nelsestuen, G. L., Resnick, R. M., Wei, G. J., Pletcher, C. H., & Bloomfield, V. A. (1981) *Biochemistry 20*, 351–358.
Nemerson, Y., & Furie, B. (1980) *CRC Crit. Rev. Biochem. 9*, 45–85.
Peters, R. (1981) *Cell Biol. Int. Rep. 5*, 733–760.
Pisarchick, M. L., & Thompson, N. L. (1990) *Biophys. J. 58*, 1235–1249.
Poglitsch, C. L., & Thompson, N. L. (1990) *Biochemistry 29*, 248–254.
Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.
Prigent-Dachary, J., Faucon, J.-F., Boisseau, M.-R., & Dufourcq, J. (1986) *Eur. J. Biochem. 155*, 133–140.
Prigent-Dachary, J., Lindhout, T., Boisseau, M.-R., & Dufourcq, J. (1989) *Eur. J. Biochem. 181*, 675–680.
Resnick, R. M., & Nelsestuen, G. L. (1980) *Biochemistry 19*, 3028–3033.
Schmidt, C. F., Zimmerman, R. M., & Gaub, H. E. (1990) *Biophys. J. 57*, 577–588.
Smith, B. A., & McConnell, H. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2759–2763.
Sui, S., Urumow, T., & Sackmann, E. (1988) *Biochemistry 27*, 7463–7469.
Suttie, J. W., & Jackson, C. M. (1977) *Physiol. Rev. 57*, 1–70.
Tendian, S. W., & Lentz, B. R. (1990) *Biochemistry 29*, 6720–6729.
Thompson, N. L., Palmer, A. G., Wright, L. L., & Scarborough, P. E. (1988) *Comments Mol. Cell. Biophys. 5*, 109–131.
Timbs, M. M., Poglitsch, C. L., Pisarchick, M. L., Sumner, M. T., & Thompson, N. L. (1991) *Biochim. Biophys. Acta 1064*, 219–228.
Wei, G. J., Bloomfield, V. A., Resnick, R. M., & Nelsestuen, G. L. (1982) *Biochemistry 21*, 1949–1959.
Wright, L. L., Palmer, A. G., & Thompson, N. L. (1988) *Biophys. J. 54*, 463–470.
Wu, J. R., & Lentz, B. R. (1991) *Biophys. J. 60*, 70–80.

Heparin Oligosaccharides Enhance Tissue-Type Plasminogen Activator: A Correlation between Oligosaccharide Length and Stimulation of Plasminogen Activation†

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ABSTRACT: The rate of plasminogen (Pg) activation by tissue-type Pg activator (t-PA) is enhanced by heparin-drived oligosaccharides. Kinetic analysis of the effects of heparin oligosaccharides, ranging in size from di- to dodecasaccharides, on Pg activation demonstrates that stimulation of the reaction is dependent on the size of the heparin oligosaccharides. Di- and tetrasaccharides enhance the activation through 2-fold increases in k_{cat} and 4-fold decreases in K_m. Hexasaccharide and larger oligosaccharides stimulate the reaction by increasing the k_{cat} by as much as 4-fold, but do not affect the K_m. Previous experiments have shown that lipoprotein(a) [Lp(a)] inhibits Pg activation by t-PA, but only in the presence of a template which enhances t-PA activity such as fibrinogen fragments or intact heparin. Similiarly, Lp(a) inhibits the enhancement of t-PA activity by the larger heparin oligosaccharides but has no effect on t-PA activity in the presence of di- and tetrasaccharides. The results of this study when considered with our previous observations (Edelberg & Pizzo, 1990) suggest that the enhancement in Pg activation by the smaller oligosaccharides is mediated exclusively via binding to t-PA while the larger oligosaccharides may interact with both t-PA and Pg. Furthermore, studies of Pg activation in the presence of both heparin oligosaccharides and fibrinogen fragments demonstrate that t-PA is stimulated preferentially by fibrinogen fragments.

Plasminogen (Pg) is a zymogen which is activated by conversion to plasmin, the enzyme which degrades fibrin clots. In the vasculature, this activation is mediated primarily by tissue-type Pg activator (t-PA) (Bachmann & Kruithof, 1984). The rate of endogenous t-PA activation of Pg is extremely slow (Hoyalerts et al., 1982) but increases in the presence of fibrinogen, CNBr fibrinogen fragments, or partially degraded fibrin (Hoyalerts et al., 1982; Rijken et al., 1982; Nieuwenhuizen et al., 1983; De Serrano et al., 1989; Harpel et al., 1985). Recent studies demonstrate that t-PA-mediated Pg activation is enhanced 5–25-fold by the glycosaminoglycan heparin (Andrade-Gordon & Strickland, 1986; Paques et al., 1986; Fears, 1988; Edelberg & Pizzo, 1990).

Heparin interacts specifically with the amino-terminal finger domain of t-PA, and thereby stimulates the rate of Pg activation (Stein et al., 1989; Edelberg & Pizzo, 1990). The finger domain is also involved in the fibrinogen fragment enhance-

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