Fluorescence Resonance Energy Transfer Study of Shape Changes in Membrane-Bound Bovine Prothrombin and Meizothrombin†

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ABSTRACT: Prothrombin activation to thrombin is a key control reaction in blood coagulation. During the process, prothrombin is sequentially cleaved at two peptide bonds (Arg^{323}−Ile and Arg^{274}−Thr) by factor X_a to generate meizothrombin and then thrombin. Phosphatidylserine (PS)-containing membranes from platelets are believed to facilitate this two-step process. Using fluorescence energy transfer (FRET), we determined the distances of closest approach between a specifically located C-terminal fluorescein of a double mutant bovine prothrombin (P(S528A, G581C)-FM) or meizothrombin (M(S528A, G581C)-FM) and phosphatidylethanolamine-*N*-rhodamine B (PE-Rh; 0−8.7 mol %) contained in membranes composed of PS (25 mol %) and phosphatidylcholine (66.3−75 mol %). Plots of the energy transfer efficiency as a function of membrane concentration, at six PE-Rh surface densities, were analyzed globally to obtain dissociation constants and binding stoichiometries as global parameters and saturating energy transfer efficiencies characteristic of each surface density. From the global analysis, the dissociation constants were estimated to be 0.32 ± 0.10 and 0.28 ± 0.12 μM with stoichiometries of 42 ± 12 and 44 ± 9 lipid/protein for prothrombin and meizothrombin, respectively. The distance of closest approach was obtained from the dependence of the saturating energy transfer efficiency on the acceptor (PE-Rh) surface density. With the assumptions of $\kappa^2 = {}^2/_3$ and $n = 1.4$, the distances were 94 ± 3 Å for prothrombin and 114 ± 2 Å for meizothrombin. Since both prothrombin and meizothrombin behave in solution as oblate ellipsoids of revolution with a long axis of 120 Å, our FRET measurements suggest that binding to PS-containing membranes induced tighter folding of the prothrombin molecule but not of the meizothrombin intermediate. This observation is consistent with our hypothesis that membrane binding plays an essential role in the sequential alignment of the bond Arg^{323}−Ile in prothrombin and Arg^{274}−Thr in meizothrombin with the active site of the membrane-bound prothrombinase in the two-step thrombin-generating process.

Thrombin is the central regulatory enzyme in the blood coagulation process. This key serine protease and regulatory molecule circulates in blood as an inactive zymogen, prothrombin (II). Activation of prothrombin to thrombin is accomplished physiologically by the prothrombinase complex, composed of factor X_a, factor V_a, Ca^{2+}, and a negatively charged phospholipid membrane surface, which is probably supplied, *in vivo*, by platelet membranes (Mann *et al*., 1988). In this complex, factor X_a serves as a serine protease to cleave two peptide bonds in prothrombin. This leads to two possible prothrombin activation pathways. One involves initial cleavage at Arg^{323}−Ile to expose the active site of the protease domain of prothrombin. This gives rise to meizothrombin as the active intermediate. The other involves initial cleavage at Arg^{274}−Thr to release the inactive protease domain, prethrombin 2, from the membrane-bound N-terminal portion of prothrombin, fragment 1.2. Previous studies have suggested that association of prothrombin and factor X_a with factor V_a on PS-containing membranes directs prothrombin activation *via* meizothrombin as the intermediate (Krishnaswamy *et al*., 1986, 1987; Armstrong *et al*., 1990). Since these studies employed factor V_a in the presence of membranes, it is unclear whether factor V_a or the membrane or both are required to direct the activation *via* the meizothrombin pathway. According both to a proposed model of prothrombin structure (Arni *et al*., 1994) and to light scattering data obtained from native bovine prothrombin (Lim *et al*., 1977), prothrombin in solution is an elongated molecule with an overall length of about 120 Å. Meizothrombin in solution was found to have an overall molecular dimension and shape similar to prothrombin in solution (Pei *et al*., 1992). In the presence of calcium ion, prothrombin and meizothrombin bind to phosphatidylserine (PS)[1]-containing membranes mainly *via* the Gla domain contained in the N-terminal fragment 1 region. The long axis of both proteins appears to be roughly perpendicular to the membrane surface with the protease domain located far from the surface (Lim *et al*., 1977; Armstrong *et al*., 1990). Similar protein−

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[1] Abbreviations: DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)-amide; ECV, *Echis carinatus* venom; S-2238, phenylalanyl-pipecolyl-arginine-*p*-nitroanilide (synthetic substrate for thrombin); SDS−PAGE, sodium dodecylsulfate−polyacrylamide gel electrophoresis; Tris, tris-(hydroxymethyl)aminomethane; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; PE-Rh, phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl); PS, bovine brain phosphatidylserine; P(S528A) and M(S528A), the active site mutant bovine prothrombin and meizothrombin; P(S528A, G581C) and M(S528A, G581C), double mutant bovine prothrombin and meizothrombin; SUV, small unilamellar vesicle.

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membrane interactions occur in some other vitamin K dependent coagulation proteins, such as factor X_a (Husten *et al*., 1987), factor IX_a (Mutucumarana *et al*., 1992), and protein C (Zhang & Castellino, 1994). When bound to a PS-containing membrane, meizothrombin appears to have interdomain interactions and secondary structure more similar to soluble as opposed to membrane-bound prothrombin (Pei *et al*., 1992). A pre-steady-state kinetic analysis suggested that association of prothrombin with PS-containing membranes enhanced the rate of cleavage by the prothrombinase at Arg^{323}−Ile but not at Arg^{274}−Thr (Walker & Krishnawamy, 1994). These observations all suggest that conformational differences between membrane-bound and solution prothrombin and meizothrombin may be crucial to the role of activated platelet membranes in blood coagulation (Pei *et al*., 1992; Lentz *et al*., 1994; Wu & Lentz, 1994).

Phosphatidylserine is located in the inner leaflet of resting platelet membranes. Upon platelet activation, PS appears in the outer leaflet of released platelet membrane vesicles (Stuart *et al*., 1995), which in turn accelerates the process of blood coagulation (Kalafatis *et al*., 1994). To test the hypothesis that membrane-induced protein conformational changes might serve to enhance the rate of prothrombin activation *via* meizothrombin, we performed fluorescence resonance energy transfer (FRET) experiments to compare the structure of prothrombin and meizothrombin upon binding to PS-containing model membranes. FRET has been used successfully to determine the topography of various membrane-bound coagulation proteins (Armstrong *et al*., 1990; Husten *et al*., 1987; Isaacs *et al*., 1986; Lu *et al*., 1989; Mutucumarana *et al*., 1992). Using a previously constructed, specifically labeled fluorescent prothrombin (P(S528A, G581C)-FM; Chen *et al*., 1996), we generated a fluorescent labeled meizothrombin (M(S528A,G581C)-FM). We showed that the fluorescence intensity of M(S528A,G581C)-FM, but not P(S528A,G581C)-FM, decreased 2% when associated with membrane, consistent with previously suggested differences between the membrane-bound conformations of these two proteins (Pei *et al*., 1992). In addition, energy transfer from the protein C-terminus to the membrane surface was more efficient for prothrombin than for meizothrombin. Using an estimate for the Förster distance of 52.5 Å, the distances from the protein C-termini to the membrane surface were calculated to be 94 ± 3 Å for prothrombin and 114 ± 2 Å for meizothrombin. This suggested that the prothrombin molecule was about 20% more compressed than meizothrombin when bound to a PS-containing membrane in the absence of factor V_a. Although we do not rule out what must be important contributions of factor V_a to prothrombin activation *in vivo*, our data are consistent with the hypothesis that binding with a PS-containing membrane helps align the bond Arg^{323}−Ile in prothrombin and subsequently the bond Arg^{274}−Thr in meizothrombin with the factor X_a active site during the two-step thrombin generation process. The sequential alignment of appropriate peptide bonds with the factor X_a active site may help direct prothrombin activation *via* meizothrombin instead of prethrombin 2.

MATERIALS AND METHODS

Materials. Bovine brain phosphatidylserine (PS),[1] 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC),[1] and phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (PE-Rh)[1] were purchased from Avanti Biochemicals (Birmingham, AL) and shown to be greater than 98% pure by thin-layer chromatography (Lentz *et al*., 1982). All lipids were stored in argon-bubbled chloroform. Solvents were low-residue, HPLC grade. Chemicals were reagent grade or better. D-Phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S2238)[1] was from Chromogenix, Molndal, Sweden. Ecarin, *Echis carinatus* snake venom (ECV), and Taipan snake venom from *Oxyuranus scutellatus* were purchased from Sigma Chemical Co. (St. Louis, MO).

As described previously (Pei *et al*., 1991; Chen *et al*., 1996), the active site mutant (P(S528A)[1]) and double mutant (P(S528A, G581C)[1]) bovine prothrombin were purified from cell culture medium of Chinese hamster ovary cells containing the cDNA encoding these mutant proteins. Prothrombin was labeled specifically at the carboxyl terminus with fluorescein maleimide and characterized as described previously (Chen *et al*., 1996). Isolation of native bovine prothrombin from serum was as described (Tendian & Lentz, 1990). Prothrombin concentrations were determined either by absorption measurements using an extinction coefficient at 280 nm of 1.44 OD $(mg/mL)^{-1}$ cm^{-1} for prothrombin (Mann, 1976) or with the bicinchonic acid assay at 560 nm (BCA kit, Pierce, Rockford, IL). The fluorescein concentration was determined by measuring the absorbance at 521 nm using an extinction coefficient of 78 OD mM^{-1} cm^{-1}. All absorbance measurements were performed at room temperature on a HITACHI U-2000 double-beam UV/vis spectrophotometer (Hitachi Instruments, Inc., Danbury, CT). The mole ratio of the probe/protein was 0.8 for all labeled prothrombin and meizothrombin used in the experiments.

Generation of Meizothrombin. We covalently linked ECV to agarose beads (Affi-gel 10, Bio-Rad, Hercules, CA) following the instructions provided by Bio-Rad. The activity of the Affi-gel-linked ECV was determined by mixing 20 μL of the ECV-Affi-gel with 60 μL of 0.5 μg/μL native prothrombin in a buffer of 20 mM Tris-HCl, 150 mM NaCl, 6 mM $CaCl_2$, pH 7.4. The reaction was stopped by centrifugation to remove the ECV-gel at intervals of 0, 30, 60, 90, and 120 min. The supernatant was then assayed for thrombin formation by S2238 as described (Chen *et al*., 1996).

To generate stable fluorescently labeled and unlabeled meizothrombin, we activated both P(S528A, G581C)-FM and P(S528A)[1] with fresh ECV-Affi-gel. As a control, native prothrombin was activated under the same conditions. Prothrombin (0.34 μg/μL) was mixed with 1/4 vol of the ECV-gel in 20 mM Tris-HCl, 150 mM NaCl, 6 mM $CaCl_2$, pH 7.4, buffer at room temperature to start the activation. At intervals of 0, 35, 60, 105, and 135 min, the sample containing native prothrombin was centrifuged to remove ECV-gel, and the supernatant was assayed with S2238. The activation of mutant prothrombin was stopped at 135 min by spinning down the ECV-gel beads. Aliquots from the supernatants were withdrawn and subjected to SDS−PAGE (10% acrylamide) under both nonreducing and reducing (3% 2-mercaptoethanol) conditions, with fluorescence being detected under UV radiation using a FOTO/PREP II fluorescent gel transilluminator (FOTODYNE, New Berlin, WI) and protein being determined by Coomassie bright blue R-250 (Sigma, St. Louis, MO). Coomassie gels and photographs of the fluorescent gels were digitized to obtain quantitative estimates of the relative amounts of proteins in

different bands using a HP ScanJet IIcx scanner (Hewlett Packard, Greeley, CO).

Preparation of Liposomes. Small unilamellar vesicles (SUV[1]) composed of PS/POPC/PE-Rh (25/66.3−75/8.7−0) were made by mixing appropriate PS, PE-Rh, and POPC stock solutions in chloroform, removing the solvent under a stream of nitrogen, and dissolving the resulting residue in cyclohexane. The dissolved lipids were shell frozen and lyophilized overnight in the dark to yield a dry powder. The dried lipids were suspended in buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) in a glass vessel and subjected to sonic disruption for 10 min, in an ice bath and under a stream of argon, using a Heat Systems ultrasonic (Farmingdale, NY, Model 350) sonicator. Following sonication, the suspension was centrifuged at 70 000 rpm for 25 min at 4 °C in a Beckman TL100 (TLA100.3 rotor) centrifuge (Beckman Instruments, Inc., Palo Alto, CA) to obtain a homogeneous suspension of vesicles (Barenholz *et al*., 1977). The top one-third of the suspension was withdrawn and maintained at room temperature in the dark until being used within 60 h. SUVs were estimated by quasi-elastic light scattering (Lentz *et al*., 1992) to have diameters in the range of 300−500 Å (mean = 394 Å), with no clear trend with lipid composition. Total phosphate concentration in a vesicle sample was determined by inorganic phosphate measurement (Chen *et al*., 1956). Rhodamine concentration was determined by absorbance measurements using an extinction coefficient at 570 nm of 80 OD mM^{-1} cm^{-1}, which was determined in the presence of 40 μM SUV by sequential addition of rhodamine B. The acceptor surface densities (σ, in rhodamine molecules/Å^2) were calculated from the experimentally determined mole ratios of rhodamine/phospholipid with the assumption that one phospholipid molecule occupied 70 Å^2 of membrane surface area (Huang *et al*., 1978).

Fluorescence Energy Transfer Measurements. We measured fluorescence spectra using an SLM 48000 MHF spectrofluorometer (SLM Aminco, Rochester, NY), equipped with a 450-W xenon lamp and monochromators in both the excitation and emission light paths (slits both 8 nm) and interfaced to a PC. The excitation shutter was closed except during scans to avoid photobleaching of the sample. All spectral measurements were carried out at room temperature in a 1 cm × 1 cm quartz cuvette (Hellma, Jamaica, NY). The buffer for all the spectral measurements was 20 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.4.

To monitor fluorescence resonance energy transfer, we followed quenching of donor (fluorescein) emission by titrating 100 nM P(S528A, G581C)-FM or M(S528A, G581C)-FM in a 800 μL vol with PS/POPC/PE-Rh SUVs (7 mM) at titrant intervals of 0, 1, 2, 4, 6, 12, 18, and 38 μL of SUVs. We excited the sample at 480 nm to minimize the direct excitation of rhodamine and scanned the donor emission from 490 to 560 nm at 1-nm intervals. The cuvette contents were constantly stirred, and emission was recorded after allowing 2 min for equilibration after each addition of lipid vesicles.

For each set of titrations, four samples were prepared in parallel. First, the blank (B), which contained 100 nM native bovine prothrombin or single mutant meizothrombin (M(S528A)), was titrated with rhodamine-free PS/POPC SUVs. Second, a sample (A), containing 100 nM native bovine prothrombin or M(S528A), was titrated with rhodamine-containing PS/POPC/PE-Rh SUVs. Third, a sample (D) containing 100 nM P(S528A, G581C)-FM or M(S528A, G581C)-FM was titrated with rhodamine-free PS/POPC SUVs. Fourth, a sample (DA) containing 100 nM P(S528A, G581C)-FM or M(S528A, G581C)-FM was titrated with rhodamine-containing PS/POPC/PE-Rh SUVs.

To compensate for any signal generated by other than the label fluorophores and, further, to compensate for emission in the DA sample from directly excited acceptor molecules, we subtracted the B signal from the D signal and the A signal from the DA signal. The corrected fluorescein emission spectra of D or DA were converted from wavelength to wavenumber and integrated from 18 000 to 20 200 cm^{-1} (495−555 nm) to obtain an integrated fluorescence intensity proportional to the relative quantum yield of donor (Q_d or Q_{da}). Assuming that the presence of the acceptor did not alter the molar absorptivity of the donor and assuming that the life time and quantum yield of the donor were unaltered by the presence of acceptor except through the energy transfer process, we normalized the dilution-corrected Q_d or Q_{da} values to the acceptor-free initial value of each titration to get Q_D or Q_{DA}, the normalized integrated relative fluorescence intensities of donor in the absence and presence of acceptor. To compensate for intermolecular energy transfer between unbound donor- and acceptor-labeled molecules in solution, we further corrected Q_{DA} by subtracting from $1/Q_{DA}$ a line with a slope obtained from regression of $1/Q_{DA}$ in the linear region (usually the last three points) of each titration. Further discussion of this correction is given in the Results (eq 8). The energy transfer efficiency (E) was then calculated as:

$$E = \frac{Q_D - Q_{DA}}{Q_D} = 1 - \frac{Q_{DA}}{Q_D} \qquad (1)$$

In the case of meizothrombin, further correction was necessary to compensate for the fluorescence due to meizothrombin des fragment 1 formed during activation of prothrombin to meizothrombin (see Results). Because of the very limited amount (∼1 mg) of labeled recombinant prothrombin available, removal of M(S528A, G581C)-FM des F1 from M(S528A, G581C)-FM would have been very difficult, if not impossible, to accomplish. Thus, we determined from densitometry that MzIIa des F1 constituted about 30% of the total protein in our meizothrombin preparation (see Results). Since the labeled MzIIa des F1 no longer contained the membrane-binding domain (Dombrose *et al*., 1979), we assumed that it did not contribute to the FRET that accompanies binding of protein to membranes. Thus, we subtracted 30% of the total fluorescence from both Q_D and Q_{DA} (after correcting Q_{DA} for nonspecific FRET) to obtain data sets to analyze in terms of our binding analysis. The net effect of this correction was to eliminate the fluorescence of MzIIa des F1 before calculating the Q_{DA}/Q_D values used in the treatment of meizothrombin binding.

Modeling and Analysis of Binding Data. To estimate binding parameters as well as energy transfer efficiencies for bound prothrombin and meizothrombin, we assumed that the corrected efficiency of membrane dependent energy transfer was related to the fraction of protein bound to sites on the phospholipid membrane:

$$P_b/P_t = \frac{E - E_0}{E_{sat} - E_0} \quad (2)$$

where P_b/P_t is the fraction of membrane-bound protein out of the total protein, E_0 and E are the corrected energy transfer efficiencies before and after addition of phospholipids, and E_{sat} is the corrected energy transfer efficiency when all the protein is bound to the membrane. With this assumption, binding can be analyzed in terms of a classical Langmuir, independent sites, adsorption model. The corrected energy transfer efficiency at any value of added total phospholipid can then be expressed in terms of the site dissociation constant for the binding reaction (K_d), the stoichiometry of binding (i), the total concentration of protein (P_t), and the total concentration of phospholipid added (PL_t) at each point in the titration:

$$E = \frac{E_{sat}}{2P_t}(PL_t i + P_t + K_d) - \frac{E_{sat}}{2P_t}\sqrt{(PL_t i + P_t + K_d)^2 - (4PL_t iP_t)} \quad (3)$$

In this equation, we assume that the corrected energy transfer efficiency is zero before addition of any phospholipid. Using this equation, we fit globally the six data sets obtained for either prothrombin or meizothrombin. The dissociation constant, K_d, and the stoichiometry, i, were assigned as the global variables (*i.e.*, common to all six sets of data in either case), and the E_{sat} values were local variables (*i.e.*, different and individually estimated for each data set). A "MULTIFIT" nonlinear least-squares regression analysis was constructed using the algorithms supplied in the SCoP package (Simulation Resources, Inc., Berrien Spring, MI). Using the "MULTIFIT", we varied K_d, i, and E_{sat} to fit simultaneously the six observed data sets to eq 3.

Calculation of the Förster Distance and the Distance of Closest Approach. The Förster distance (R_0) is the distance at which the efficiency of energy transfer is 50%. It has a characteristic value for any given fluorophore pair, given by:

$$R_0 = 9.78 \times 10^3 (n^{-4}\phi_d\kappa^2 J)^{1/6}\ (\text{Å}) \quad (4)$$

where R_0 is in Å and κ^2 is a geometric factor that depends on the relative orientations of the donor and acceptor transition dipoles (taken as $^2/_3$, see Results). For our situation, fluorescein and rhodamine separated by a protein-rich environment, n (the refractive index of the environment) is taken as 1.4, ϕ_d is the integrated fluorescence intensity of the fluorescein in the absence of rhodamine, and J is the normalized spectral overlap integral, calculated from observed rhodamine (acceptor) absorption and fluorescein (donor) emission spectra according to:

$$J = \frac{\int F_d(\lambda)\epsilon_a(\lambda)\lambda^4\, d\lambda}{\int F_d(\lambda)\, d\lambda} \quad (5)$$

where J has units of M^{-1} cm^3, $F_d(\lambda)$ is the fluorescence intensity of fluorescein in the absence of rhodamine at wavelength λ, and $\epsilon_a(\lambda)$ is the molar absorption coefficient of rhodamine B at λ. Finally, the quantum yield of the donor in the absence of acceptor, ϕ_d, was determined as follows (Parker and Rees, 1966):

$$\frac{\phi_{d1}}{\phi_{d2}} = \frac{Q_{d1}}{Q_{d2}}\cdot\frac{A_2}{A_1} \quad (6)$$

where ϕ_{d1} is the quantum yield of P(S528A, G581C)-FM or M(S528A, G581C)-FM in our buffer (20 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.4) and in the presence of sufficient acceptor-free membranes to bind more than 95% of the protein. ϕ_{d2}, which was estimated to be 0.92 (Weber & Teale, 1957), is the quantum yield of disodium fluorescein in 0.1 M NaOH. Q_{d1} and Q_{d2} are the integrated areas of the corrected emission spectra of, first, P(S528A, G581C)-FM or M(S528A, G581C)-FM in the presence of membranes and, second, disodium fluorescein solution, respectively. A_1 and A_2 are the absorbances at 490 nm for P(S528A, G581C)-FM or M(S528A, G581C)-FM at appropriate phospholipid concentrations and of disodium fluorescein solution, respectively.

Generally, L is defined as the distance of closest approach between two parallel infinite planes containing uniformly and randomly distributed energy donor or acceptor molecules. Assuming each protein has the same conformation, all protein-bound donor probes occupy a plane above the membrane plane. We measure L as the distance of closest approach between the protein-bound donor and the acceptor at the membrane surface. According to the theory of energy transfer between two parallel infinite planes, each with uniformly and randomly distributed energy donor or acceptor molecules, respectively (Dewey & Hammes, 1980), L is given by the expression:

$$\left(\frac{Q_D}{Q_{DA}}\right)_{sat} = 1 + \frac{\pi\sigma}{2}\cdot\frac{R_0^6}{L^4} \quad (7)$$

where σ is the surface density of energy acceptor in rhodamine/Å^2, and $(Q_D/Q_{DA})_{sat}$ was calculated from the values of E_{sat} (eq 1). Plotting the $(Q_D/Q_{DA})_{sat}$ versus σ, we obtained the slopes and calculated the value of L with the determined R_0.

RESULTS

Meizothrombin Generation. Native meizothrombin undergoes autolysis (Doyle & Mann, 1990; Pei & Lentz, 1991) to form meizothrombin des fragment 1 (MzIIa des F1). To obtain stable meizothrombin for structural analysis, we constructed an active site mutant bovine prothrombin (P(S528A); Pei *et al.*, 1991) and a double mutant bovine prothrombin with a specific C-terminal fluorescent label (P(S528A, G581C)-FM; Chen *et al.*, 1996). Both the unlabeled and labeled recombinant prothrombins with single and double mutants were characterized as globally native-like, properly activated, but without activity toward either itself or S2238,[1] which is a synthetic substrate specific for thrombin (Pei *et al.*, 1991; Chen *et al.*, 1996).

ECV is generally used to generate meizothrombin from native prothrombin in the presence of excess amounts of a specific thrombin inhibitor, DAPA[1], to prevent meizothrombin autolysis. Since the recombinant prothrombin has no autolysis activity, we tried to generate labeled and unlabeled meizothrombin from P(S528A, G581C)-FM and P(S528A) with ECV[1]-gel in the absence of DAPA. After 2.5 h

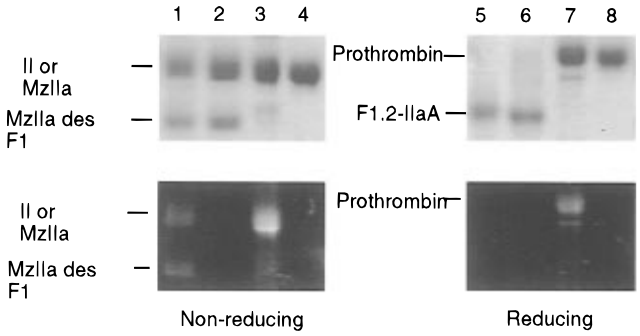

FIGURE 1: Meizothrombin generation from P(S528A) and P(S528A, G581C)-FM by ECV-gel. P(S528A) and P(S528A, G581C)-FM (0.34 mg/mL) were incubated with $^1/_4$ vol of ECV-Affi-gel for 135 min at room temperature; 10-μL aliquots of these reaction mixtures were subjected to SDS−PAGE under both nonreducing (lanes 1−4 at left) and reducing (lanes 5−8 at right) conditions. The upper panel is the SDS−PAGE gel stained with Coomassie blue; the lower panel shows the same gel photographed using a fluorescence transilluminator. Lanes indicated are 1 and 5, M(S528A, G581C)-FM; 2 and 6, M(S528A); 3 and 7, P(S528A, G581C)-FM; 4 and 8, P(S528A).

incubation with $^1/_4$ vol of ECV-gel at room temperature, all prothrombin in the reaction was converted to meizothrombin or, unexpectedly, to MzIIa des F1. We show elsewhere that the MzIIa des F1 was generated during meizothrombin preparation by a thrombin-like activity of ECV instead of by meizothrombin autolysis (Chen *et al*., 1997).

Shown in Figure 1 are SDS−PAGE gels in which labeled and unlabeled prothrombin and meizothrombin migrated at the same positions under nonreducing conditions (lanes 1−4). Under reducing conditions (lanes 5−8), prothrombin migrated more slowly due to reduction of disulfide bonds. Meizothrombin, as expected, migrated as fragment 1.2 linked with thrombin A chain and thrombin B chain (not visible on the gel). The fluorescent label was previously shown to be located in the thrombin B chain of the thrombin molecule (Chen *et al*., 1996) and thus is not present in lane 5 (reduced gel of double mutant meizothrombin). The lower band in lane 1 (the labeled, double mutant meizothrombin) demonstrates the presence of labeled MzIIa des F1-like peptide in the reaction. It was impossible to eliminate MzIIa des F1-like peptide, since halting ECV activation sooner resulted in incomplete prothrombin proteolysis. Thus, we had to choose between minimizing the presence of either prothrombin or meizothrombin des fragment 1-like peptide in our meizothrombin preparations. Since meizothrombin des fragment 1-like peptide should not bind to a membrane, we allowed ECV catalysis to run sufficiently long to convert all prothrombin to either meizothrombin or meizothrombin des fragment 1-like peptide. This resulted in our preparation containing roughly 28% MzIIa des F1 as detected by densitometry of Coomassie-stained gels or 32% by quantitation of fluorescence gel photographs. The meizothrombin des fragment 1-like peptide formed in the reaction was then taken into account during data analysis as described in Materials and Methods.

Fluorescence Resonance Energy Transfer from Protein C-Terminus to Membrane Surface. Fluorescence energy transfer from protein-bound fluorescein to rhodamine on the membrane surface was shown by demonstrating a decrease in fluorescein fluorescence and an increase in rhodamine fluorescence when P(S528A, G581C)-FM and M(S528A, G581C)-FM were titrated with rhodamine-labeled membranes. The emission spectra of both donor (fluorescein) and acceptor (rhodamine) were recorded, as shown in Figure 2, from 490 to 630 nm. Addition of PS/POPC/PE-Rh SUV to P(S528A, G581C)-FM (spectrum b) decreased the fluorescence peak at 521 nm (spectrum a). As a demonstration that this decrease resulted from FRET rather than from some other quenching mechanism, an increase in the fluorescence intensity of rhodamine is shown as spectrum f in the insert of Figure 2. Because the decrease in donor fluorescence could be more accurately followed than could the increase in acceptor fluorescence, FRET was followed by monitoring this donor fluorescence decrease.

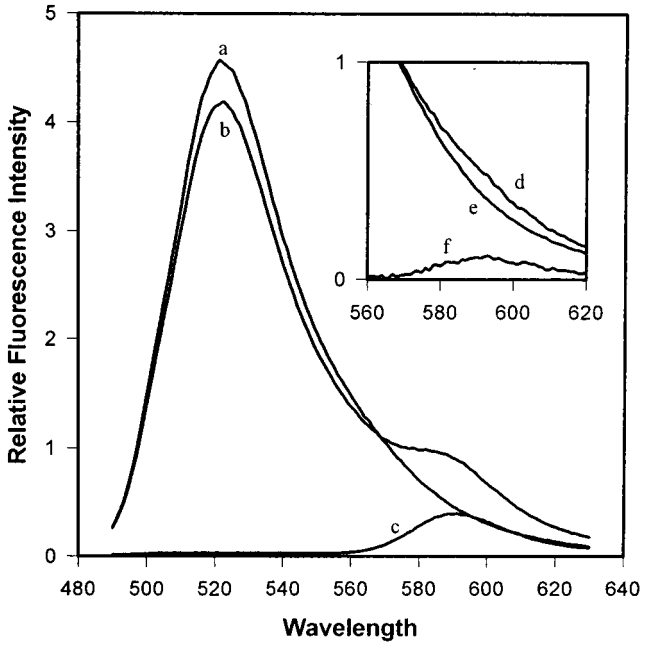

FIGURE 2: Energy transfer from fluorescein-labeled protein to rhodamine-labeled membrane. P(S528A, G581C)-FM was examined in the presence and absence of PS/PC/PE-Rh SUV (25/74/1). Spectra consist of the emission of both donor (fluorescein, peak at 521 nm) and acceptor (rhodamine, peak at 590 nm). Corrected emission spectra are shown for (a) P(S528A, G581C)-FM; (b) P(S528A, G581C)-FM with 46 μM PS/PC/PE-Rh SUVs, and (c) 46 μM PS/PC/PE-Rh SUVs. The inert shows a blowup of the rhodamine portion of the spectrum, in which spectrum **d** was obtained by subtracting spectrum c from b, spectrum e was obtained by adjusting the intensity of spectrum a to that of spectrum b at 550 nm, and spectrum f was obtained as the difference between spectra d and e. The spectrum f demonstrates the increase in rhodamine fluorescence due to energy transfer from fluorescein.

Prothrombin and Meizothrombin Respond Differently to Association with Membranes. Previous work from our laboratory suggested that PS-induced differences between membrane-bound prothrombin and meizothrombin might be crucial to the role of platelet membranes in blood coagulation (Pei *et al*., 1992; Lentz *et al*., 1994; Wu & Lentz, 1994). To compare directly the effect of PS-containing membranes on these two proteins, we titrated P(S528A, G581C)-FM and M(S528A, G581C)-FM with PS/POPC (25/75) membranes in the presence of 5 mM Ca^{2+}. The integrated fluorescence intensities were determined and normalized as described in Materials and Methods. Plots of the normalized integrated fluorescence intensities (Q_D) of P(S528A, G581C)-FM and M(S528A, G581C)-FM as a function of lipid concentration are shown in Figure 3. The normalized integrated fluorescence intensities of labeled meizothrombin decreased about 2% in the presence of membranes, reaching a limiting value

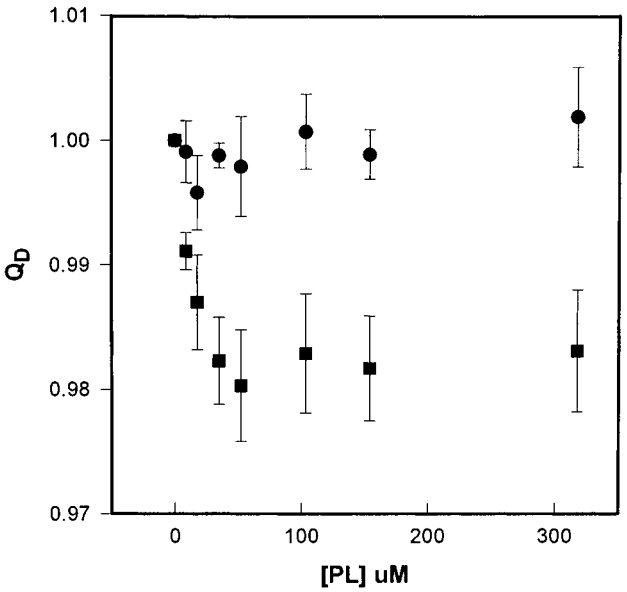

FIGURE 3: Difference in binding of P(S528A, G581C)-FM and M(S528A, G581C)-FM to PS membrane. P(S528A, G581C)-FM (●) and M(S528A, G581C)-FM (■) were titrated with PS membranes, which contained no PE-Rh. Corrected emission spectra were integrated and normalized to obtain relative integrated fluorescence intensities of the donor (Q_D) for each titration point.

around a phospholipid concentration of 40 μM. In contrast, there was no such decrease observed in labeled prothrombin in response to titration with phospholipids. This observation indicates that the C-terminal probe experienced a slight change in its environment on binding of meizothrombin to a PS-containing membrane, while there was no such change on binding of prothrombin. This result is consistent with our previous observation that interdomain interactions and secondary structure were altered in different ways in meizothrombin relative to prothrombin when these very similar proteins bound to membranes (Pei *et al*., 1992).

Steady-State FRET Studies with Fluorophore-Labeled Membranes. We monitored the decrease in integrated relative fluorescence intensity of P(S528A, G581C)-FM and M(S528A, G581C)-FM in the presence of increasing concentrations of acceptor-containing membranes (Q_{da}). The acceptor-containing membranes were composed of 0−8.7% PE-Rh, 25 mol % PS, and 66.3−75 mol % POPC. To compare the changes of integrated fluorescence intensity generated from various titrations, we normalized the integrated fluorescence intensity of donor (Q_{da}) obtained at each membrane concentration with the integrated fluorescence intensity of donor before addition of membrane for each of the titrations. Normalized integrated fluorescence intensity of donor is referred to as Q_{DA} and is plotted as a function of added membrane concentration in Figure 4A.

Wu and Brand (1994) have pointed out that energy transfer between donor and acceptor groups attached to the same molecule has two components, namely, intramolecular and intermolecular energy transfer. In a system such as ours, which involves interactions between donor and acceptor groups attached to separate molecules, energy transfer will always be intermolecular but will still have two components, saturable and nonsaturable energy transfer. The saturable component, on which this report focuses, is due to specific binding interactions of donor-labeled protein to the acceptor-labeled membranes. Nonsaturable energy transfer occurs

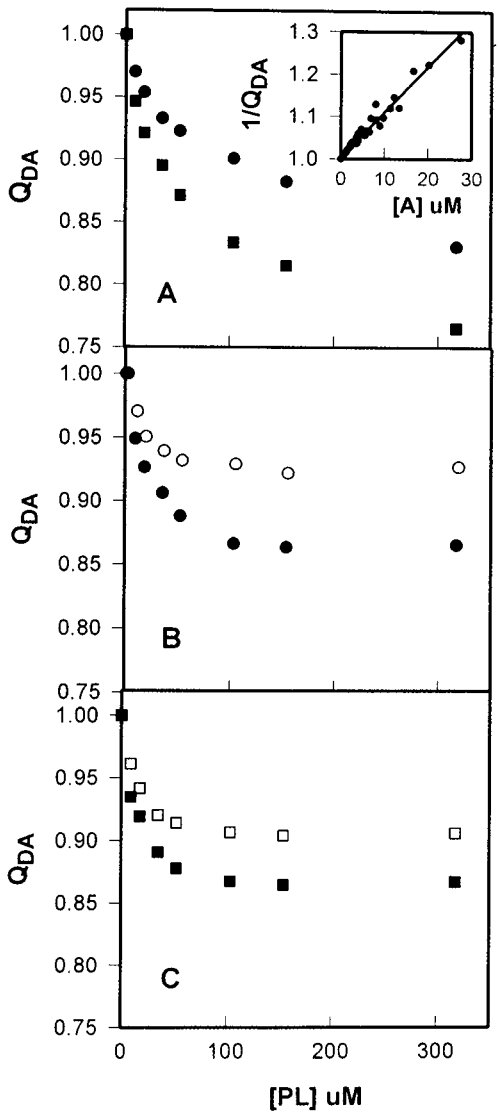

FIGURE 4: Changes in integrated fluorescence intensities of P(S528A, G581C)-FM and M(S528A, G581C)-FM as a function of increasing lipid and acceptor concentrations. P(S528A, G581C)-FM (circles) and M(S528A, G581C)-FM (squares) were titrated with membranes containing 0−8.7% PE-Rh, 25 mol % PS, and 66.3−75 mol % POPC. Corrected emission spectra were integrated to obtain relative integrated fluorescence intensities of the donor for each titration point. A: Normalized integrated fluorescence intensities (Q_{DA}) at acceptor surface densities of 3.6×10^{-4} and 5.1×10^{-4} Rh/Å^2 for P(S528A, G581C)-FM (●) and M(S528A, G581C)-FM (■), respectively. The insert shows the data from the three highest lipid concentrations in all 12 titrations, demonstrating that the energy transfer between unbound donor- and acceptor-labeled molecules is a linear function of acceptor concentration. B and C: Normalized, integrated fluorescence intensities (Q_{DA}) corrected for nonsaturable energy transfer (see Results) as a function of concentration of PS/POPC membranes containing PE-Rh. Panel B represents fluorescein-labeled prothrombin titrated at acceptor surface densities of 2.1×10^{-4} (○) and 3.6×10^{-4} (●) Rh/Å^2. Panel C represents fluorescein-labeled meizothrombin titrated at acceptor surface densities of 5.1×10^{-4} (□) and 9.1×10^{-4} (■) Rh/Å^2.

between protein-bound donors and acceptors attached to protein-free membranes. The unsaturable component led to a continuous, linear decrease in Q_{DA} even when the phospholipid concentration was increased beyond that necessary to bind all the protein in a sample based on published binding constants (Pei *et al*., 1992; Cutsforth *et al*., 1989; Figure 4A). In order to define the saturable component, we had to

correct for the unsaturable component. The ratio of Q_{DA}/Q_D for nonsaturable energy transfer in solution is given as a function of total acceptor concentrations [A] by Förster (1959) as:

$$Q_D/Q_{DA} = 1 + \frac{\beta}{[A_0]} \times [A] \quad (8)$$

where $[A_0]$ is the critical transfer concentration corresponding to an average of one acceptor molecule in a sphere of radius R_0, and β is a constant determined by donor concentration and the R_0 for the pair. Since our situation involves acceptor molecules distributed randomly on the surface of membrane vesicles that are distributed randomly in solution, we expected that the basic functionality of eq 8 should hold but that β would have to be established experimentally. This equation indicates that Q_D/Q_{DA} should be a linear function of total acceptor concentration [A] at supersaturating membrane concentrations. Since Q_D is a constant close to 1 in all the cases considered here, $1/Q_{DA}$ vs [A] for nonsaturable energy transfer should be a straight line through the point (0,1) with a slope of $\beta/[A_0]$. The decrease in Q_{DA} with increasing membrane concentration shown in Figure 4A was induced by both saturable and nonsaturable energy transfer. The insert to Figure 4A, which includes the data from the three highest lipid concentrations of each of our 12 titrations, demonstrates the linear relationship between $1/Q_{DA}$ and [A]. This linear relationship is as predicted by eq 8 and indicates that the continued decrease in the donor-integrated fluorescence intensity at acceptor concentrations beyond binding saturation was mainly due to nonspecific energy transfer from donor-labeled protein to acceptor-labeled membranes to which the protein was not physically bound. An inner filter effect would also yield such a linear functionality, although this should be very minor, since acceptor absorbance was insignificant (<0.03 at 480 nm).

Using the method described above, we corrected all plots of Q_{DA}, such as shown in Figure 4A, by subtracting the linear dependence of $1/Q_{DA}$ on total acceptor concentration shown in the insert of Figure 4A. This procedure yields the plots in Figure 4B,C, in which the decrease of Q_{DA} with membrane concentration reflects only the saturable energy transfer due to reversible binding of donor-labeled protein to acceptor-labeled membrane for prothrombin and meizothrombin, respectively. As can be seen in panels B and C of Figure 4, the responses of donor fluorescence to increasing phospholipid concentrations and to different acceptor surface densities (σ) were similar for prothrombin and meizothrombin. However, for both prothrombin and meizothrombin, the integrated donor fluorescence intensity decreased to smaller limiting values upon binding to membranes with a higher acceptor surface density.

Energy transfer efficiency (E, eq 1) is most conveniently used to describe the data from an energy transfer experiment. This is because the energy transfer efficiency is expected to be proportional to the fraction of protein bound to membranes containing PE-Rh (eq 2). Using the classical, independent-sites, Langmuir adsorption model expressed in eq 3, we analyzed in a global fashion energy transfer titration curves obtained at six acceptor densities for either prothrombin or meizothrombin. The globally determined dissociation constants (K_d) and stoichiometries ($1/i$) for both prothrombin and meizothrombin are summarized in Table 1, along with

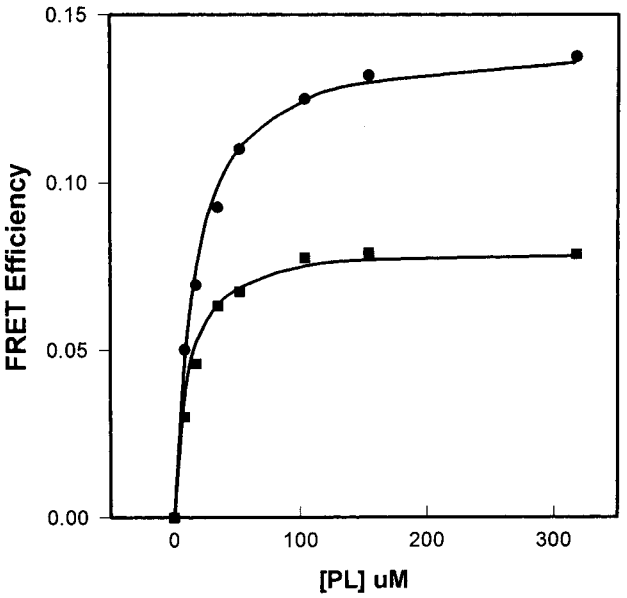

FIGURE 5: Fluorescence energy transfer from P(S528A, G581C)-FM and M(S528A, G581C)-FM to the membrane surface. Fluorescence energy transfer efficiencies (E) of P(S528A, G581C)-FM (●) and M(S528A, G581C)-FM (■) as a function of membrane concentration are compared at acceptor surface densities of 3.6×10^{-4} and 5.1×10^{-4} Rh/Å^2, respectively. Lines drawn through the data points indicate the best fits obtained from the global fit of six such titration curves as described in Materials and Methods.

Table 1: Summary of the Dissociation Constants (K_d), Stoichiometries (i), and Saturated Energy Transfer Efficiency Values (E_{sat}) at Various Acceptor Surface Densities

K_d (M)	$1/i$	σ (Rh $\times 10^{-4}$/Å^2)	E_{sat}
	Prothrombin		
$(3.2 \pm 1) \times 10^{-7}$	42 ± 12	1.2	0.046
		1.6	0.057
		1.9	0.075
		2.1	0.083
		3.1	0.093
		3.6	0.144
	Meizothrombin		
$(2.8 \pm 1.2) \times 10^{-7}$	44 ± 9	3.6	0.070
		5.1	0.080
		5.5	0.088
		7.5	0.121
		9.1	0.129
		12.4	0.208

the saturated energy transfer efficiency (E_{sat}) determined at each acceptor surface density. As can be seen, there was no significant difference in the membrane-binding parameters (K_d or $1/i$) between prothrombin and meizothrombin, which agrees with our earlier report that both proteins bind with $K_d = 0.37$ μM and $1/i = 45$ PL/site (Pei *et al.*, 1993). On the other hand, prothrombin displayed more efficient energy transfer at low acceptor surface densities than meizothrombin did even at higher acceptor surface densities. The solid lines drawn through the data in Figure 5 illustrate the fit obtained at two particular acceptor surface densities by this global analysis procedure. The values of E_{sat} determined in this way were used to assess the distances of closest approach (L) between donor on the protein and acceptor on the membrane surface for prothrombin and meizothrombin, respectively.

Distance of Closest Approach. According to eq 7, the distance of closest approach (L) of the protein-bound donor

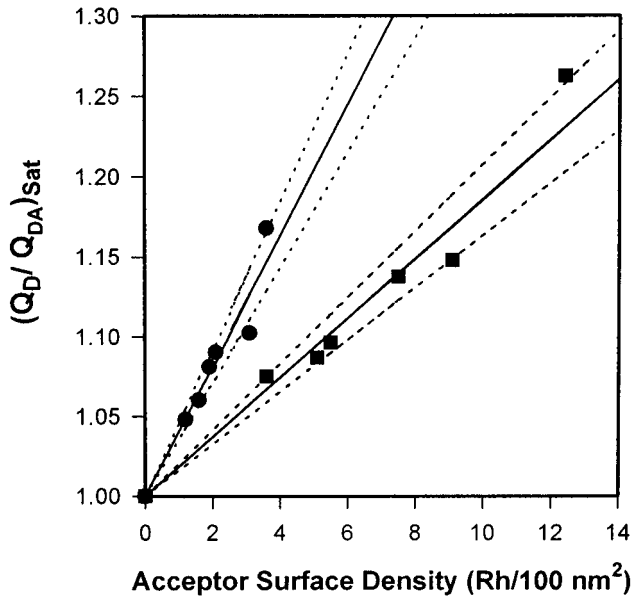

FIGURE 6: $(Q_D/Q_{DA})_{sat}$ as a linear function of the acceptor surface densities (σ). $(Q_D/Q_{DA})_{sat}$ was calculated from E_{sat}, the energy transfer efficiency values at saturating membrane concentrations. Shown here are the plots of $(Q_D/Q_{DA})_{sat}$ vs σ prothrombin (●) and meizothrombin (■) with their linear regressions (—) to give slopes as 421 ± 80 and 198 ± 21 Å^2/Rh. Dotted lines are drawn to indicate the regions of 95% confidence in these slopes of the fitted lines. The differences in these slopes, and thus in the protrusion lengths of prothrombin and meizothrombin above the membrane surface, are thereby seen to be statistically significant.

to the surface-located acceptor is related to the quantum yield ratio (given, under our conditions, by Q_D/Q_{DA}) for membrane-bound protein. We converted E_{sat} values to values of $(Q_D/Q_{DA})_{sat}$ using eq 1, and Figure 6 presents plots of $(Q_D/Q_{DA})_{sat}$ as a function of σ, the acceptor surface density in units of Rh/100 nm^2 (or 10^{-4} Rh/Å^2). Linear regression analysis of these data yielded slopes of 421 ± 80 and 198 ± 27 Å^2/Rh for prothrombin and meizothrombin, respectively. Regions of 95% confidence in these slopes are shown as the areas between dotted lines in Figure 6. This significant difference in slopes confirms that prothrombin has a conformation on a PS-containing membrane different from that of meizothrombin. We assume that the donor molecules on the protein C-termini and the acceptor molecules on the membrane surface were, for the purpose of calculating the orientation factor (κ^2), nearly randomly oriented, an assumption supported by the polarization values measured for these two probes, 0.230 ± 0.004 and 0.235 ± 0.006, respectively. This assumption leads to an estimate of κ^2 close to $^2/_3$. Since the polarization values are lower than 0.3 for both donor and acceptor molecules, the errors in R_0 values introduced by this assumption are likely to be less than 10% (Lackowicz, 1983; Haas *et al*., 1978). Using measured emission and excitation spectra, $\kappa^2 = {}^2/_3$, and $n = 1.4$, R_0 was calculated to be 52.5 Å for both P(S528A, G581C)-FM and M(S528A, G581C)-FM. Based on this R_0, L was calculated to be 94 ± 3 Å for prothrombin and 114 ± 2 Å for meizothrombin.

To obtain the plots in Figure 6, we had to make several corrections to the data. Three of these corrections (background fluorescence, nonsaturable FRET, and normalization) were straightforward and routine. The fourth was not routine but was necessitated by the fact that MzIIa des F1 was unavoidably formed during preparation of meizothrombin, even meizothrombin prepared from active site mutant prothrombin. Since MzIIa des F1 does not bind to membranes, we corrected for its contribution by subtracting its fluorescence intensity from the observed total fluorescence, as described in Materials and Methods. It is worth commenting on the extent to which this correction affects our conclusion. If this correction had not been made, the slopes shown in Figure 6 would have been 421 ± 80 and 127 ± 27 Å^2/Rh, leading to values of 94 ± 3 and 127 ± 2 Å for L of prothrombin and meizothrombin, respectively. Thus, while the uncertainty associated with heterogeneity in the meizothrombin preparation can alter the absolute value of L, this uncertainty does not affect our main conclusion that the overall distance from the C-terminus to the membrane for prothrombin is significantly smaller than that for meizothrombin.

DISCUSSION

Validity of the Method. Fluorescence resonance energy transfer (FRET) has been applied as a "spectroscopic ruler" to measure the conformational difference between prothrombin and meizothrombin when bound to a PS-containing membrane. The distances of closest approach between the protein C-termini and the membrane surface were estimated to be 94 Å for prothrombin and 114 Å for meizothrombin. Obviously, the absolute accuracy of the calculated values of L depends heavily on the value of R_0, which has to be estimated from spectroscopic properties of the donor and acceptor (Wu & Brand, 1994). Although uncertainty in the value of R_0 may cause up to a 10% uncertainty in the calculation of L, the slopes of $(Q_D/Q_{DA})_{sat}$ vs acceptor surface densities in Figure 6 are unaffected by this uncertainty. The clear difference in the measured slope values indicates that prothrombin assumes a configuration very different from that of meizothrombin upon binding to a PS-containing membrane. This result is consistent with a previous report that meizothrombin and prothrombin are not equivalent substrates for the pre-steady-state cleavage at R^{274}—T by prothrombinase (Walker & Krishnaswamy, 1994).

The distances we determined are average distances between the protein-bound donors and acceptors at the outer surface of the phospholipid bilayer. However, the extent of FRET could be influenced also by the presence of acceptor molecules located in the inner leaflet of the vesicle bilayer. From eq 7, the ratio of Q_D/Q_{DA} is a function of the fourth order of L. The phospholipid bilayers are about 50-Å thick (Isaac *et al*., 1986), which is almost equal to R_0 for our donor—acceptor pair. Thus, acceptor chromophores at the inner leaflet of the vesicle bilayer are about $3R_0$ away from the donor at the protein C-terminus. As the acceptors on the outer leaflet are about $2R_0$ from donor chromophores, the contribution from acceptors on the inner leaflet is likely to be less than one-fifth of the observed energy transfer. If we were to correct for this effect, our estimates of the overall lengths of proteins would be about 10 Å shorter than the numbers we have estimated for both prothrombin and meizothrombin. Thus, this estimation can alter the absolute values of L, but it does not affect the 20% difference in the overall lengths of prothrombin and meizothrombin when bound to a PS-containing membrane.

Our FRET measurements utilized different ranges of acceptor surface densities for prothrombin and meizothrombin. The acceptor surface densities, in units of Rh/10000 Å^2,

were 1.2−3.6 for prothrombin and 3.6−12.4 for meizothrombin. This difference was necessary to compensate for the substantial difference in the overall lengths of prothrombin and meizothrombin when bound to a PS-containing membrane. As indicated in eq 7, the efficiency of energy transfer depends on acceptor surface density as well as on the distance between donor and acceptor molecules. Because 5% energy transfer efficiency is about the minimum needed to obtain a reasonably precise donor−acceptor distance (Dewey & Hammes, 1980), we performed FRET on meizothrombin at the higher acceptor densities partly to meet this criterion. As discussed by Dewey and Hammes (1980), eq 7 gives the limiting value of Q_D/Q_{DA} when $L \gg R_0$. A smaller ratio of L/R_0 requires smaller acceptor surface densities in order for eq 7 to be valid. Thus, we adjusted acceptor surface densities for prothrombin and meizothrombin measurements both to optimize the precision of these measurements and to assure the validity of eq 7.

To generate liposomes with different acceptor surface densities, we incorporated 0−8.7 mol % PE-Rh with 25 mol % PS and an appropriate amount of POPC. From our global analysis, the different amounts of phosphatidylethanolamine used in different experiments had little influence on prothrombin or meizothrombin binding with membranes containing 25 mol % PS. Smirnov and Esmon (1994) also reported that the incorporation of up to 40 mol % phosphatidylethanolamine into 20 mol % PS-containing membranes did not affect prothrombin activation by the prothrombinase. Although Billy *et al*. (1995) reported that incorporation of 20 mol % phosphatidylethanolamine into 5 mol % PS-containing membranes dramatically increased the assembly of prothrombinase complex and the rate of prothrombin activation, these authors stressed that phosphatidylethanolamine contributes to thrombin generation only when membranes contained less than 10 mol % PS. Since 25 mol % PS was used in all our FRET experiments, phosphatidylethanolamine likely had little effect on the protein−membrane interactions we detected.

Comparison with Results in the Literature. The dissociation constants and stoichiometries determined here for prothrombin and meizothrombin binding to 25/75 bovine PS/POPC SUV are similar to the values (0.5 ± 0.02 μM, 51 ± 8) we have reported at similar ionic conditions for binding of prothrombin to 20/80 PS/POPC SUV (Cutsforth *et al*., 1989). Meizothrombin binding has not been described under the ionic conditions used here, but in agreement with the results obtained here, we have shown that it binds to PS-containing membranes with the same affinity as prothrombin (Pei *et al*., 1992). This excellent agreement with results obtained by a very different method (light scattering) argues strongly for the validity of the assumptions made in analyzing the data presented here.

Unlike prothrombin, meizothrombin is an active serine protease with its active site located in the C-terminal half of the molecule. Our results show that a fluorescein located in the catalytic domain of meizothrombin is 114 Å distant from rhodamine at the phospholipid surface. A crude model of prothrombin (Arie *et al*., 1994) would place our fluorescein probe at the extreme opposite end of the prothrombin molecule from the γ-carboxyglutamic residues thought to be involved in membrane binding (Ratcliffe *et al*., 1993). We have reported previously that thermal denaturation profiles of membrane-bound meizothrombin were similar to those of prothrombin in solution (Pei *et al*., 1992). The two molecules were also reported to have the same hydrodynamic shapes in solution (Pei *et al*., 1992). Another FRET measurement showed that the meizothrombin active site was 71 Å above the membrane surface (Armstrong *et al*., 1990). This is consistent with our measurement and, in conjunction with the other results we have quoted, suggests that the active site of meizothrombin is located roughly two-thirds of the distance between the C-terminal end of the molecule and the membrane surface. This agrees with the model proposed by Arni *et al*. (1994), in which the three amino acids forming the active site were located at about three-fifths of the distance between the N- and C-termini of the molecule (Arni *et al*., 1994). Thus, it would seem that membrane binding does not induce significant changes in the shape of the meizothrombin molecule, consistent with the report of Pei *et al*. (1992).

In contrast to meizothrombin, prothrombin appears to undergo substantial conformational and shape changes upon binding to a PS-containing membrane. Membrane-induced changes in secondary structure have been demonstrated (Wu & Lentz, 1991). Thermal denaturation studies showed that the conformational change involved alterations both in domain structures and in interdomain interactions between fragment 1, fragment 2, and prethrombin 2 (Lentz *et al*., 1991, 1994). Using quasi-elastic light scattering (QELS), Lim *et al*. (1977) estimated the lengths of these three domains as 38, 30, and 45 Å. Thus, the overall length of prothrombin in solution should be about 113 Å, the sum of the lengths of its three domains. This is consistent with the estimate (120 Å) of Arni *et al*. (1994). Our estimation of a 94 Å distance of closest approach from fluorescein at the C-terminus of prothrombin to rhodamine at the phospholipid surface suggests that binding with PS membranes induces the prothrombin molecule either to "fold up" or to tilt with respect to the bilayer normal, while the meizothrombin molecule does not. Given the very similar stoichiometries of binding of prothrombin and meizothrombin to a PS-containing membrane surface (Pei *et al*., 1992; Table 1 of this work), both molecules seem to cover a similar surface area of the membrane, approximately 20 lipid molecules in the contacting leaflet. If we view prothrombin as an oblate ellipsoid of revolution with a minor axis of 20 Å radius (Österberg *et al*., 1980), it would cover roughly 20 lipid molecules if bound in an orientation with the long axis perpendicular to the membrane surface. This suggests that prothrombin and meizothrombin do not differ substantially in their tilt relative to the membrane surface, rather both seem to bind roughly perpendicular to the surface. Thus, prothrombin seems to "fold up" internally to achieve the shorter fluorescein-to-rhodamine distance that we have measured relative to that of meizothrombin. This is consistent with the observation that membrane binding alters substantially interdomain interactions within the prothrombin molecule (Lentz *et al*., 1991, 1994) but probably not within the meizothrombin molecule (Pei *et al*., 1992).

Hypothesis for the Functional Role of Membrane-Induced Different Conformations of Prothrombin and Meizothrombin. A hypothetical model is proposed in Figure 7 to suggest the role that these membrane-induced conformational changes may play in the two-step thrombin generation process. We have measured a 20% difference between prothrombin and meizothrombin in the distance from the C-termini of these

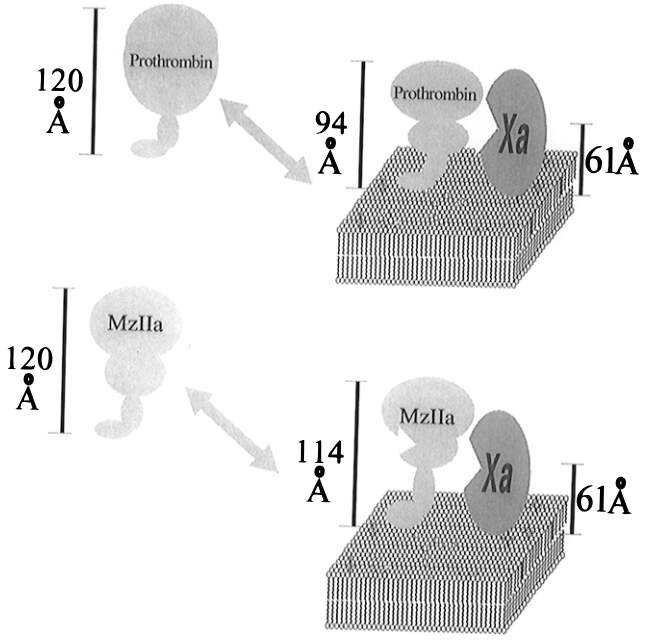

FIGURE 7: Schematic model. The overall lengths of both prothrombin and meizothrombin in solution are reported to be about 120 Å (Arni *et al*., 1994; Lim *et al*., 1977); the distances of closest approach between the probes on the protein C-terminus and the membrane surface were estimated to be 94 ± 3 and 114 ± 2 Å for prothrombin and meizothrombin, respectively. The active site of factor X_a is reported to be 61 Å above the membrane surface in the absence of factor V_a (Husten *et al*., 1987). The membrane-induced conformational changes demonstrated here have the potential to align appropriate peptide bonds in prothrombin and meizothrombin with the factor X_a active site.

two proteins to the membrane surface. This suggests that binding to PS-containing membranes induces tighter folding of the prothrombin molecule but subsequent extension of the meizothrombin intermediate. Our previous prothrombin thermal denaturation studies suggested a membrane-binding-induced substantial linkage between the fragment 1, fragment 2, and prethrombin 2 domains and involved a direct interaction of fragment 2 with the membrane (Lentz *et al*., 1994). In the context of the current results, this might suggest that fragment 2 acts as a hinge in modulating the configuration of the membrane-bound substrate and intermediate. Since the active site of membrane-bound factor X_a is 61 and 69 Å above the membrane surface in the absence and presence of factor V_a, respectively (Husten *et al*., 1987), the membrane-binding-induced structural difference between the substrate and the intermediate suggests that membrane binding may help to sequentially align the bond Arg^{323}−Ile in prothrombin and Arg^{274}−Thr in meizothrombin with the factor X_a active site in the two-step thrombin generation process. This suggestion is in agreement with the previous observation that membrane binding increased about 60-fold the pre-steady-state rate of cleavage at Arg^{323}−Ile, while binding increased only about 5-fold the rate of the second cleavage at Arg^{274}−Thr (Walker & Krishnaswamy, 1994). Thus, we suggest that the membrane-induced structural changes demonstrated here are crucial in directing prothrombin activation to thrombin *via* the meizothrombin rather than the prethrombin 2 intermediate. Papers by Krishnaswamy *et al*. (1986, 1987) suggested that the association with factor V_a on PS-containing membranes was crucial for prothrombin activation *via* meizothrombin as the intermediate. However, since both PS-containing membranes and factor V_a were present in the kinetic experiments performed by Krishnaswamy *et al*., it remains uncertain whether factor V_a or PS-containing membranes are responsible for directing the pathway of prothrombin activation. Our results suggest that PS-containing membranes produce structural changes in prothrombin and meizothrombin that could at least partially explain the kinetic results of Krishnaswamy *et al*. Interactions of substrate and/or enzyme with factor V_a may provide additional effects that direct the selection of the prothrombin activation pathways. PS-induced changes in factor X_a activity (Koppaka *et al*., 1996) could also have effects on the selection of the activation pathway.

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