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Kinetic and Hydrodynamic Analysis of Blood Clotting Factor V–Membrane Binding†

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ABSTRACT: The kinetics and hydrodynamic properties of factor V–membrane interaction were characterized. Factor V bound to membranes containing acidic phospholipids with a high collisional efficiency. For membranes of 20% phosphatidylserine–80% phosphatidylcholine, an association rate constant of $(1.13 \pm 0.10) \times 10^8$ M^{-1} s^{-1} was obtained. These membranes contained about 20 factor V binding sites per vesicle of 3.6×10^6 daltons. This association rate represented about a 30% collisional efficiency. Dissociation of factor V was measured by a fluorescence energy transfer method with a dissociation rate constant of 0.0055 s^{-1} at 10 °C. The equilibrium dissociation constant for binding to these membranes at 10 °C and 0.14 M ionic strength was 5×10^{-11} M. Ionic strength, pH, calcium, and charge density in the membrane had large effects on the rate of factor V–membrane dissociation, indicating a strongly ionic interaction between protein and membrane. In contrast, the association rate was nearly insensitive to ionic strength. The membrane-binding properties were relatively unchanged after thrombin digestion of factor V or after long-term protein storage which resulted in loss of procoagulant activity. Other proteins of the prothrombinase reaction greatly decreased the rate of factor Va–membrane dissociation. At protein saturation, factor V increased the hydrodynamic radius of phospholipid vesicles by 11.4 nm. In contrast, factor Va increased the hydrodynamic vesicle radius by only about 5 nm. The mass of membrane-bound protein was comparable for both proteins.

Blood clotting factors Va and Xa plus phospholipids form the enzymatic complex, prothrombinase, which converts prothrombin to thrombin [see Jackson & Nemersen (1980) for a review]. The substrate and enzyme of this reaction, prothrombin and factor Xa, are vitamin K dependent proteins and, in the presence of calcium, bind to membranes containing acidic phospholipids. Both the equilibrium and dynamic properties of these protein–membrane interactions have been studied extensively [see Wei et al. (1982) and references therein].

Factor V also associates with membranes. It is known that the factor V–membrane interaction requires acidic phospholipids but not calcium (Jobin & Esnouf, 1967; Bloom et al., 1979). Factor V binding to purified phospholipids (Bloom et al., 1979) appeared quite different from binding to platelets (Tracy et al., 1981), the presumed relevant biological membrane. Proteolysis by thrombin converts factor V to factor Va, a much more active form of the protein. The membrane-binding properties of factor V appeared to differ from those of factor Va (Bloom et al., 1979). Factor Va has been shown to enhance the binding of factor Xa to platelets or phospholipid vesicles (Miletich et al., 1978; Dahlbäck & Stenflo, 1978; Tracy et al., 1979). This tighter binding probably arises from simultaneous protein–protein and protein–membrane interactions.

The properties of factors V– and Va–membrane interaction were investigated further in order to better characterize this interaction and to determine the relative affects of protein activation on this association. Kinetic analysis revealed a rapid association, suggesting a facile protein–phospholipid recognition. No major differences between factors V and Va were apparent. Hydrodynamic measurements showed a major difference in the degree to which these proteins protrude from the membrane surface.

Materials and Methods

Highly purified phospholipids were purchased from Sigma Chemical Co. Single bilayer vesicles of defined composition were prepared by sonication and gel filtration according to standard procedures (Nelsestuen & Lim, 1977; Huang, 1969). Phospholipid concentration was determined by phosphate assay (Chen et al., 1956) using a phosphorus to phospholipid weight ratio of 25. Unless indicated, the buffer used was 0.05 M Tris–0.1 M NaCl (pH 7.5).

Dansyl-PE[1] was formed from 3 equiv of dansyl chloride and 1 equiv of dipalmitoylphosphatidylethanolamine in chloroform

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[1] Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; dansyl, 8-(dimethylamino)-1-naphthalenesulfonyl; dansyl-PE, *N*-dansylphosphatidylethanolamine; Tris, tris(hydroxymethyl)aminomethane; Dnp, dinitrophenyl; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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containing 4 equiv of triethylamine (Waggoner & Stryer, 1970). The mixture was stirred at room temperature for 48 h, and the product was isolated on a silica gel column washed with chloroform (2 volumes) and then eluted with chloroform–methanol–water (65:25:4). Phosphorus determination and absorbance at 340 nm ($\epsilon = 4.3 \times 10^3$; Weber, 1952) indicated a dansyl/PE ratio of 0.97 in the product. Fluorescence measurements were made in a Perkin-Elmer 44A fluorescence spectrophotometer equipped with a circulating water bath to maintain temperature.

The molecular weight of phospholipid vesicles and protein–vesicle complexes was determined from 90° light-scattering intensity by using the method and apparatus previously described (Pletcher et al., 1980). This method is valid for particles with dimensions small compared to the wavelength of light. Angular dependence indicated that the molecular weight of the vesicles estimated by 90° light scattering deviated only a small degree from the molecular weight obtained by extrapolation to 0 scattering angle (Wei et al., 1982). The dimensions of the protein–vesicle complex are somewhat greater, but angular dependence of molecular weight showed about the same error. This error should be constant over the association curve and was not corrected in these studies.

Light-scattering intensity was used to calculate membrane-bound protein by the method of Nelsestuen & Lim (1977). Briefly, excess light scattering from a solution of protein and phospholipid is attributable to protein–membrane binding. The relative intensity measurements can be used to obtain the molecular weight ratio from the relationship $I_{s2}/I_{s1} = [(\partial n_2/\partial c_2)^2(M_{r_2}/M_{r_1})^2]/(\partial n_1 \partial c_1)^2$, where M_{r_2} is the molecular weight of the protein–phospholipid vesicle complex, M_{r_1} is the molecular weight of the original vesicles, I_s is the light-scattering intensity, and $\partial n/\partial c$ is the refractive index increment. If the weight concentration of vesicles is known, the weight concentration of membrane-binding protein can be obtained.

Association rate constants for factor V–membrane binding were obtained from analysis of 90° light-scattering intensity changes following protein–vesicle mixing in a stopped-flow apparatus. The apparatus and methods of analysis were described previously (Wei et al., 1982). Experimental observations were compared to theoretical curves generated according to eq 8 in Wei et al. (1982). The rate constant producing the best fit to the experimental data was obtained. The rate constant is expressed on the basis of protein-binding sites. Comparison to protein–vesicle collisions must therefore consider n, the estimated number of binding sites per vesicle (Wei et al., 1982). The data discriminated the rate constant to about ±10%. That is, greater than 10% deviation from the best-fit value was unacceptable. The rate of dissociation of factor V from the membrane was so slow (see below) that it could be set at 0 for modeling the association data.

Diffusion constants and Z-averaged hydrodynamic radii of vesicles and protein–vesicle complexes were obtained by quasi-elastic light scattering by using the methods and apparatus previously described (Pletcher et al., 1980).

Factor V activity was determined by clotting time assay using purified reagents. One unit corresponded to the factor V activity in 1 mL of bovine plasma. One hundred microliters of a solution consisting of 15 mg/mL fibrinogen, 1.0 mg/mL phospholipid (20% PS), and 0.1 mg/mL prothrombin, in 0.05 M Tris–0.1 M NaCl (pH 7.4), was warmed to 37 °C. Fifty microliters of factor V solution and 50 μL of buffer (37 °C) were added, and the assay was initiated by 10 μL of factor Xa solution (30 μg/mL) plus 100 μL of buffer containing 10 mM $CaCl_2$. The time required to form a fibrin clot was measured.

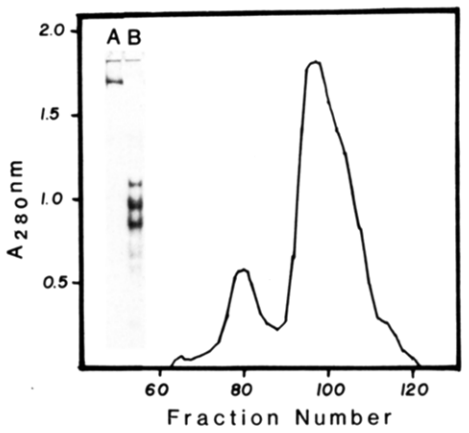

FIGURE 1: Elution of factor V from Bio-Gel A-1.5m. The QAE-Sephadex eluate obtained from 2.5 L of plasma was applied to a 3 × 110 cm column of gel and eluted with buffer. The peak eluting prior to the major protein peak contained the factor V activity. The stained gels are (A) the purified factor V (reduced) and (B) thrombin-digested factor V (reduced). The two largest peptides in gel B had molecular weights of about 100 000 and 80 000.

Bovine factor V was prepared by using variations of methods previously described (Esmon, 1979; Nesheim & Mann, 1979). Bovine blood was collected by venipuncture into 0.1 M sodium oxalate–50 mM benzamidine hydrochloride–20 mM PMSF (9:1, v/v). All buffers used in purification contained 2 mM Ca^{2+} and 2 mM benzamidine. Fifty milligrams of $BaSO_4$ was added per mL of plasma (usually 2.5 L per preparation), and the mixture was stirred for 0.5 h at room temperature. Following centrifugation, poly(ethylene glycol) 6000 was added to the supernatant to a concentration of 4% (w/v). After the supernatant was stirred at 4 °C for 1 h, the precipitate was removed by centrifugation. The supernatant was diluted with equal parts distilled H_2O and buffer. QAE-Sephadex was added (5 g of dry gel/L of original plasma), and the suspension was stirred for about 45 min at room temperature. The adsorbed QAE-Sephadex gel was recovered by vacuum filtration and washed 5–7 times by resuspension in 0.5 (original plasma) volume buffer and filtration. The gel was packed into a column (4 × 30 cm), and factor V was eluted with buffer containing 0.4 M NaCl. The fractions containing activity were pooled, and the protein was precipitated with ammonium sulfate (final concentration of 65% saturation, 4 °C). After centrifugation, the precipitate was dissolved in about 30 mL of buffer and chromatographed on a 3 × 110 cm column of Bio-Gel A-1.5m (Bio-Rad Laboratories) equilibrated at 4 °C. The factor V activity peak eluted prior to the main protein peak (Figure 1). The resulting preparations were often highly pure by the criterion of $NaDodSO_4$ gel electrophoresis. The single band observed in gel A (Figure 1) was very similar in molecular weight to preparations of factor V obtained by Nesheim & Mann (1979). The factor V protein was sensitive to thrombin digestion (Figure 1, gel B) and produced major peptides at about 100 000, 80 000, and 55 000 daltons. The former two correlate closely with the active peptides of factor Va (Esmon, 1979).

An additional step included in some preparations was chromatography on dextran sulfate–Sepharose 4B prepared by the method of Pepper & Prowse (1977). The protein eluted from QAE-Sephadex was applied directly to a 2 × 30 cm column of dextran sulfate–Sepharose at 4 °C and washed with 200 mL of buffer containing 0.4 M NaCl. The factor V activity was eluted with 0.4 M NaCl–30% saturated ammo-

nium sulfate or with buffer containing 0.7 M NaCl. The active fractions were then applied directly to the Bio-Gel A-1.5m column. The final specific activity was about 70 units of factor V/mg of protein which corresponded to about a 4000-fold purification of factor V activity from plasma. This purification could be completed in 24–30 h, and the protein was usually used within 24 h. Thrombin digestion of factor V was performed with a 1:100 (w/w) protein ratio at 37 °C for 10 min.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the method of Weber & Osborn (1969) with 5% acrylamide tube or slab gels. Molecular weights were estimated in the usual manner by using myosin (200 000), transferrin (85 000), bovine serum albumin (68 000), and unreduced fibrinogen (340 000) standards. Slab gels were stained for protein with silver stain (Merril et al., 1981). Total protein concentrations were estimated from absorbance at 280 nm by using an $E^{1\%}_{280nm}$ of 9.6 for factor V (Nesheim et al., 1979).

Prothrombin, factor X, factor Xa, and prothrombin fragment 1 were prepared and quantitated by standard methods used previously (Nelsestuen & Lim, 1977).

Results

Factor V–Membrane Binding at Equilibrium. Figure 2 shows analyses of protein–membrane binding measured by 90° light-scattering intensity. As protein was added to the vesicles, the molecular weight of the complex increased until apparent saturation was achieved. The M_{r_2}/M_{r_1} value at the plateau provided the maximum weight ratio of protein in the complex. This was used to calculate the value of n, the number of factor V binding sites per vesicle. The weight-average molecular weight of the vesicle was determined experimentally, and the molecular weight used for factor V was 330 000 (Nesheim et al., 1979).

At low protein to phospholipid ratios, the binding curve was nearly linear, and the percentage of membrane-bound protein did not vary with the concentration of phospholipid (Figure 2A and inset). If unbound protein were in equilibrium with bound protein, this percentage should vary with phospholipid in the manner shown for prothrombin (Figure 2B). The constancy of bound factor V indicated (a) that the dissociation constant for membrane binding was much lower than the concentration of binding sites and (b) that the unbound protein in Figure 2A (inset) did not have membrane-binding capability. Some factor V preparations obtained early in these studies were not homogeneous by the criterion of $NaDodSO_4$ gels and showed less than quantitative membrane binding (e.g., Figure 2A inset). Many factor V preparations that appeared highly pure still showed less membrane-binding protein than total protein estimated by 280-nm absorbance. In all cases the weight concentration of membrane-binding protein was estimated from the initial slope of the binding curve (Figure 2A). This analysis proved to be consistent and allowed highly reproducible association constants regardless of apparent protein homogeneity (see below). All factor V preparations used in this study contained at least 40% membrane-binding protein.

The amount of membrane-bound factor V was unaffected by addition of calcium (10 mM) or EDTA. However, the complex could be dissociated by addition of 1–2 M salt, which could be used to demonstrate the reversibility of binding. While digestion of factor V with thrombin had relatively little effect on the amount of membrane-bound protein (see below), EDTA caused considerable dissociation of thrombin-digested protein from the membrane (data not shown). This is consistent with the report that EDTA causes dissociation of factor

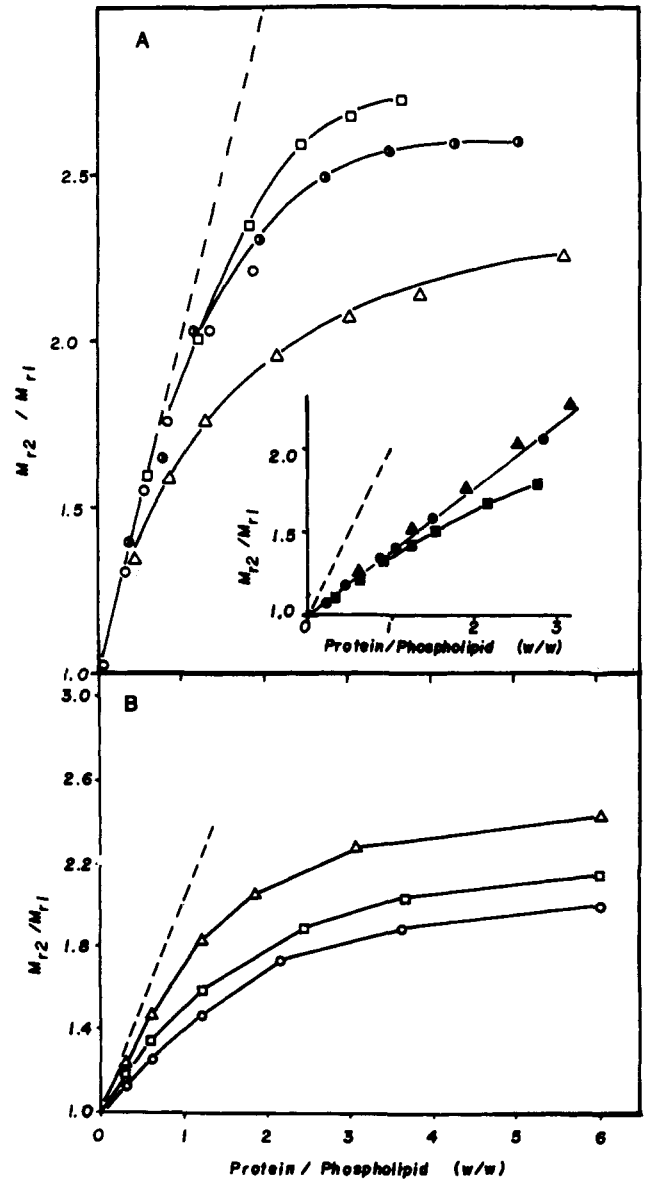

FIGURE 2: Measurement of factor V–membrane binding by light scattering. Phospholipid vesicles were assigned a relative molecular weight of 1 (M_{r_1}). The relative molecular weight of the protein–membrane complex (M_{r_2}) was obtained from analysis of light-scattering intensity. Part A shows studies with two factor V preparations. The phospholipids used include PS/PC (10/90) [11.1 (△) and 63.0 μg/mL (■)], PS/PC (20/80) [11.8 (□), 91.5 (○), and 30.5 μg/mL (▲)], and PG/PC (40/60) [92.5 (●), and 12.3 μg/mL (◑)]. Part B shows an experiment using prothrombin and vesicles of PS/PC (20/80) [23 (○), 46 (□), and 93 μg/mL (△)] in buffer containing 3 mM calcium. The dashed lines represent the curves that would be obtained if all of the added protein bound to the membrane.

Va peptides (Esmon, 1979; Hibbard & Mann, 1980).

Stopped-Flow Light-Scattering Analysis of Factor V–Membrane Binding. Figure 3 shows light-scattering intensity changes after mixing factor V and suitable phospholipid vesicles. The smooth curves show theoretical results for factor V–membrane binding at the concentrations and rate constants given. These were generated according to the method of Wei et al. (1982) and assume a binding model with noninteracting sites on the membrane. The fit of experimental and theoretical curves appeared good. A 3-fold variation in protein and phospholipid concentrations gave the same rate constants (data not shown).

Replicate stopped-flow tracings gave highly reproducible rate constants (range <10%). Comparison studies using the

Table I: Factor V-Membrane Binding Kinetics at 10 ± 1 °C[a]

phospholipid vesicles				binding parameters			collisional
composition	$2R_h$ (nm)	M_r	n	k_1 (M^{-1} s^{-1})	k_2 (s^{-1})	K_D (M)	efficiency[c]
PS/PC (5/95)	30	3.6×10^6	6	1.1×10^8			10%
PS/PC (10/90)	29	3.2×10^6	10	$1.1 \pm 0.05 \times 10^8$[b]	0.026	2.6×10^{-10}	19%
PS/PC (15/85)	30	3.6×10^6	18	0.7×10^8			
PS/PC (20/80)	30	3.5×10^6	18	$1.13 \pm 0.10 \times 10^8$	0.0055	5×10^{-11}	35%
PS/PC (20/80) + thrombin digestion			18	$1.7 \pm 0.3 \times 10^8$	0.0046	2.7×10^{-11}	50%
PG/PC (40/60)	24.4	2.4×10^6	12	1.3×10^8	0.020	1.5×10^{-10}	25%

[a] The values were determined by methods outlined in the text. [b] Association rate constant errors are standard deviations given only when three or more independent determinations (see text) were run. [c] Collisional efficiency = nk_1 divided by collisional rate constant. The latter was calculated from hydrodynamic radii and diffusion constants of the protein and vesicles by Smoluchowski theory.

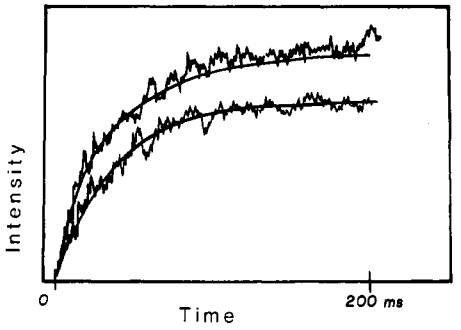

FIGURE 3: Stopped-flow analysis of factor V–membrane binding. Phospholipid [PS/PC (20/80); 200 μg/mL after mixing) and factor V (200 μg/mL after mixing, upper curve; 160 μg/mL after mixing, lower curve) were mixed in the stopped flow, and the 90° light-scattering intensity was monitored. The experiments were performed at 10 °C in 0.05 M Tris (pH 7.5)–0 M NaCl (upper curve) and in 0.05 M Tris (pH 7.5)–0.4 m NaCl (lower curve). The smooth curves are best-fit theoretical curves generated for the concentrations given, 18 binding sites per vesicle of 3.6×10^6 daltons, and association rate constants of 0.7×10^8 M^{-1} s^{-1} (upper curve) and 0.6×10^8 M^{-1} s^{-1} (lower curve).

same protein and phospholipid preparations (e.g., temperature effects) therefore should be accurate within this limit. Independent analyses used different protein and phospholipid preparations. Seven such independent analyses conducted under similar conditions over a 1-year period (10 ± 2 °C, 20% phosphatidylserine; Table I) gave an average association rate constant of $(1.13 \pm 0.10) \times 10^8$ M^{-1} s^{-1} (standard deviation). This included apparently homogeneous protein as well as preparations of lower purity. Data interpretations assumed similar uncertainties for independent analyses. Factor Va gave a larger standard deviation for three independent analyses (Table I). Factor Va was precipitated by the vacuum degassng procedure (Wei et al., 1982). To avoid this problem, factor V was degassed and then digested with thrombin to form Va.

The results in Table I demonstrate that the association rate constant was relatively unaffected by the charge density in the membrane (compare 5–40% acidic phospholipid). Since the rate constant was expressed per binding site, the collisional efficiency was approximately proportional to the concentration of acidic phospholipid in the membrane. The rate constant was also relatively unaffected by ionic strength; the changes in total intensity in Figure 3 were approximately equal to differences in protein concentration. The temperature dependence of the association rate constant gave an activation energy of 6.5 kcal/mol (Figure 4A). Thrombin digestion of factor V increased its association rate somewhat (Table I) which may be due to the smaller radius and higher diffusion constant for factor Va (Esmon, 1979).

Dissociation Rates. The fluorescence emission spectrum of factor V and the excitation spectrum of dansyl-PE overlap

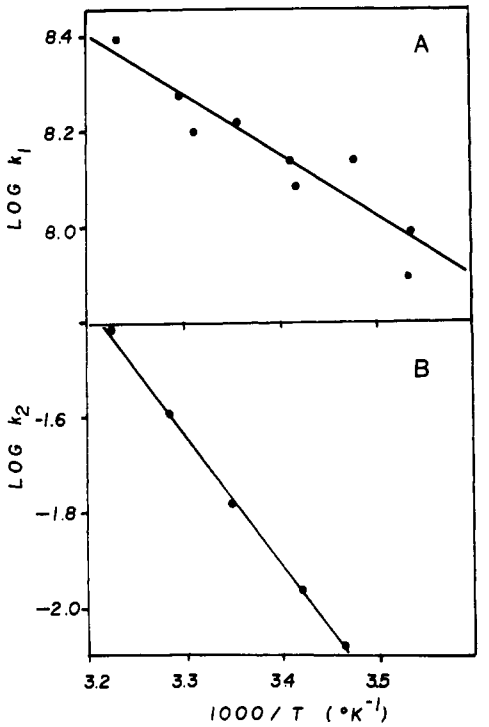

FIGURE 4: Temperature dependence of association and dissociation rate constants. Part A gives the Arrhenius plot for association rate constants obtained with two different factor V preparations. The activation energy was estimated to be 6.5 kcal/mol. Part B shows the dissociation rate constants where the activation energy was estimated to be 13 kcal/mol.

so that fluorescence energy transfer can occur when distance and orientation requirements are met. This was accomplished when factor V bound to phospholipid vesicles containing 2–5% dansyl-PE (F_0; Figure 5). Control experiments demonstrated that unbound protein provided no detectable energy transfer from protein fluorescent groups to dansyl groups. Prothrombin and factor X did not produce detectable energy transfer even when bound to these vesicles. When expressed on a relative basis, the amount of energy transfer correlated closely with the amount of membrane-bound factor V estimated by changes in light-scattering intensity (Figure 5, inset). In subsequent calculations the fluorescence intensity due to energy transfer was assumed to be proportional to the amount of membrane-bound protein.

The experiment shown in Figure 5 illustrates the method used to measure factor V–membrane dissociation. A large excess of nonfluorescent phospholipid was added at zero time to effectively trap all of the factor V dissociating from the fluorescent vesicles. First-order rate plots of the resulting fluorescence decay (Figure 6) showed only slight deviation

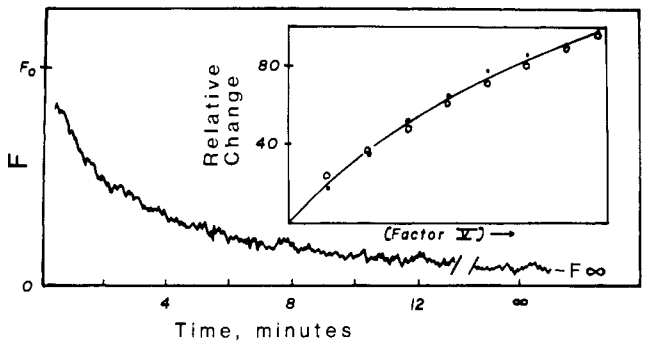

FIGURE 5: Factor V–membrane dissociation measured by fluorescence energy transfer. Phospholipid (20 μg of PS/PC/dansyl-PE (18/80/2)) and factor V (30 μg) were mixed in 1.5 mL of 0.05 M Tris–0.05 M NaCl (pH 7.5) at 20 °C. Fluorescence intensity (F_0) at 510 nm (excitation at 280 nm) was measured. Fluorescence from direct excitation of the dansyl moiety by excitation light has been subtracted so only fluorescence due to energy transfer (F) is shown. At zero time 200 μg of phospholipid (PS/PC, 20/80) was added. F_∞ is the fluorescence at equilibrium measured by mixing both phospholipids before adding the factor V. The inset shows a comparison of M_{r_2}/M_{r_1} [(O) determined as in Figure 2A] with fluorescence intensity due to energy transfer (●) as factor V was added. Both experiments used the same phospholipid and are expressed relative to the apparent maximum for each curve.

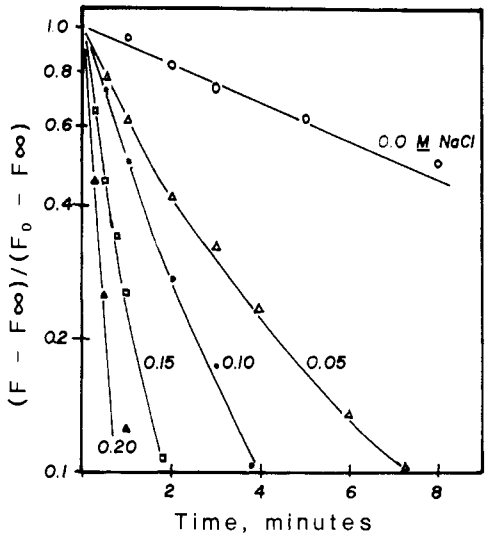

FIGURE 6: First-order rate plots of factor V–membrane dissociation. Fluorescence intensity due to energy transfer was used to determine the dissociation rate at 20 °C. The identities of F, F_0, and F_∞ are given in Figure 5. The dissociation rates were obtained in 0.05 M Tris buffer containing the amounts of NaCl indicated.

from linearity. The first reaction half-time was used to obtain the single dissociation rate constant reported (Table I). All of the studies presented here were repeated on two preparations of factor V. One preparation was highly pure on the basis of $NaDodSO_4$–acrylamide gel electrophoresis, and the other was clearly contaminated. The results were indistinguishable.

Dissociation rates at different ionic strengths indicated a great sensitivity to salt (Figure 6). Potassium chloride produced similar effects. Calcium had a large effect on the dissociation rate even when the total ionic strength was approximately constant. In one comparison, 2 mM calcium increased the dissociation rate by 4-fold when the solution ionic strength was maintained at 0.14 M. The effect of calcium was saturable, with the half-maximal effect at about 0.5 mM calcium. This corresponded closely to the apparent dissociation constant for calcium–phosphatidylserine binding under similar conditions (K_D = 0.44 mM; Nelsestuen & Lim, 1977). Furthermore, the dissociation rate was strongly affected by

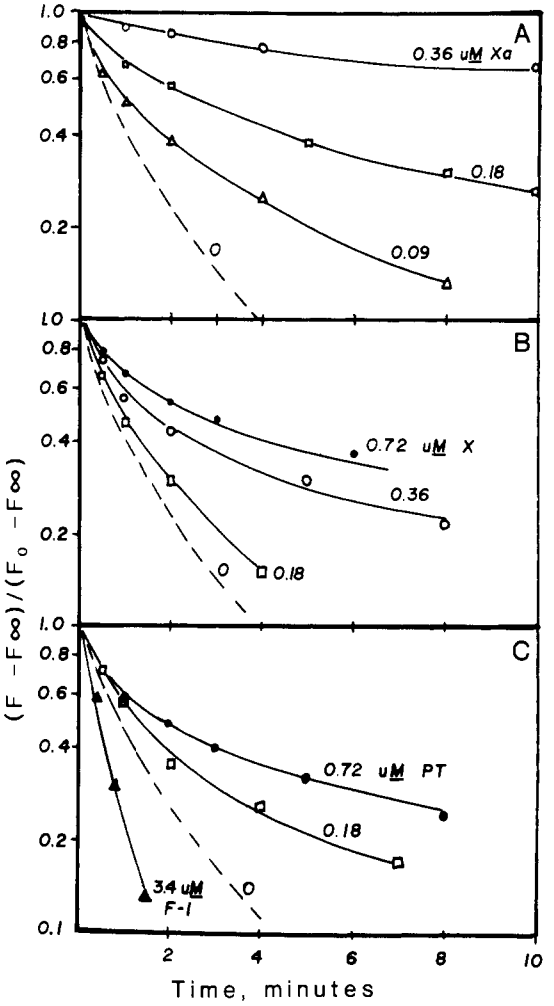

FIGURE 7: Effects of prothrombinase-related proteins on factor Va–membrane dissociation. These experiments were conducted at 25 °C by the method shown in Figure 5 in buffer containing 2 mM calcium. The factor Va concentration was about 60 nM, and the phospholipid (PS/PC/dansyl PE, 20/75/5) was 20 μg/1.5 mL. The dashed line in each part shows dissociation of factor Va alone. The experiments contained the concentrations of factor Xa (part A), factor X (part B), prothrombin (PT, part C) and prothrombin fragment 1 (F-1, part C) shown.

the density of phosphatidylserine in the membrane (compare 10 and 20% PS, Table I). All of these observations indicated that dissociation of factor V from the membrane was strongly affected by the charge density at the membrane surface.

Temperature effects on the rate of factor V–membrane dissociation from these membranes indicated an activation energy of 13 kcal/mol (Figure 4B). Phosphatidylglycerol was somewhat less efficient in factor V–membrane binding than was phosphatidylserine (Table I). Thrombin digestion of factor V had a small but reproducible effect on dissociation of factor V from the membrane (Table I). The dissociation rate was constant between pH 5.5 and 8. Above pH 8.5 the rate of dissociation increased dramatically (data not shown).

Control experiments indicated that the dansyl moiety in the fluorescent vesicles did not have a measurable effect on the dissociation rate. This was shown by the complement of the experiment in Figure 5 where dissociation of factor V from unlabeled vesicles was monitored by subsequent association with labeled vesicles.

Effects of Other Membrane-Bound Proteins on Factor Va–Membrane Dissociation Rates. Factor Xa had a very large effect on the rate of dissociation of thrombin-digested factor V from the membrane (Figure 7A). Substantial effects

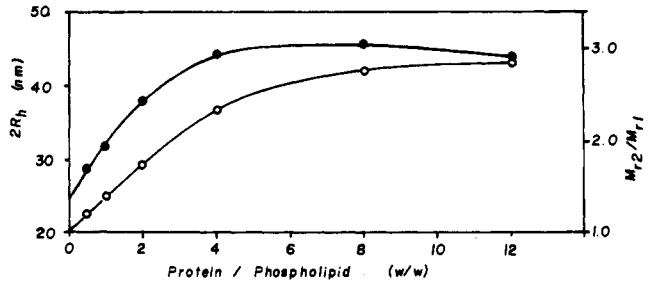

FIGURE 8: Effect of factor V on the hydrodynamic radius of phospholipid vesicles. The phospholipid concentration was maintained at 110 μg/mL PG/PC (40/60). M_{r_2}/M_{r_1} (○) was determined from light-scattering intensity measured in the quasi-elastic light-scattering apparatus and was corrected for contributions from free protein. The hydrodynamic radius (R_h) of the particles (●) was determined by quasi-elastic light scattering.

Table II: Hydrodynamic Properties of Factor V–Membrane Complexes

phospholipid vesicles		plus saturating protein		
composition	$2R_h$ (nm)	$2R_h$ (nm)	M_{r_2}/M_{r_1}	ΔR_h
PS/PC (20/80)	28.5	51.0	3.0	11.2
thrombin treated	28.5	38.0	3.0	4.6
PG/PC (40/60)	24.4	45.8	2.8	11.4
thrombin treated	24.4	32.2	2.7	3.9

are observed at stoichiometric amounts of factor Xa, and higher levels of Xa provided essentially irreversible binding of factor Va to the membrane on this time scale. Prothrombin and factor X also slowed the rate of factor Va–membrane dissociation, but much higher concentrations were needed for similar effects (Figure 7B,C). Calculations from these data suggested that the Xa interaction was 2 to 3 orders of magnitude higher than the factor X interaction. In contrast, prothrombin fragment 1, the membrane-binding region of prothrombin, increased the rate of factor Va–membrane dissociation (Figure 7C). The effects of all of these proteins were dependent on calcium. Factor Xa, factor X, and prothrombin had no effect on the rate of dissociation of intact factor V from the membrane; thrombin digestion was essential for these effects.

Hydrodynamic Changes. Figure 8 shows the *Z*-averaged particle diameters for phospholipid vesicles at various protein to phospholipid ratios. As factor V protein was added to the vesicles, their average size changed in an orderly fashion and reached a maximum radius change of about 110 Å. As indicated previously (Lim et al., 1977), the protein actually protrudes further than this from the membrane surface. Thrombin digestion reduced the particle radius considerably (Table II) without noticeable reduction in the amount of membrane-bound protein (M_{r_2}/M_{r_1}, Table II).

Effects of Protein Storage. The procoagulant activity of some of the less pure factor V preparations decreased with storage at 0 °C. The most highly purified preparations of factor V retained full biological activity for over 2 months. Even when procoagulant activity was lost, however, the membrane-binding function remained constant. This was true for preparations which had lost over 90% of procoagulant activity during prolonged storage. The dissociation and association rate constants appeared unaffected as did the weight concentration of membrane-binding protein. The basis for loss of biological activity in these preparations was not documented but did not appear to be due to a thrombin-like degradation; the inactive protein gave a vesicle radius change of 9.5 nm (like factor V, Table II) which was reduced to 4.5 nm (like factor Va) upon thrombin treatment. The membrane-binding function of factor V appeared to be relatively independent of procoagulant activity and of thrombin activation.

Discussion

Independent analysis of association and dissociation rates reported here indicate that factor V–membrane interaction conforms reasonably well to a simple bimolecular binding process with noninteracting protein binding sites on the membrane and single association and dissociation rate constants:

$$P_iV + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P_{i+1}V \quad (i = 0, \ldots, n-1)$$

where P is protein and V is the phospholipid vesicle containing *n* factor V binding sites. The dissociation rate constant is first order, and its determination did not require knowledge of the value of *n* nor the absolute concentration of either protein or phospholipid.

The association rate constant was obtained from stopped-flow light-scattering analysis by using methods developed for prothrombin (Wei et al., 1982). Accurate estimation of this second-order rate constant required values for the protein and phospholipid concentrations and *n*. The concentration of membrane-binding protein was estimated from membrane-binding analysis (Figure 2A) rather than from 280-nm absorbance. The concentration of free factor V at equilibrium was not measurable, so equilibrium constants and the number of binding sites could not be obtained from Scatchard or double-reciprocal plots. The value of *n* was estimated from the maximum weight of factor V bound to the vesicles (see Figure 2A) and the equilibrium constants were obtained from forward and reverse rate constants.

Bloom et al. (1979) also studied the equilibrium binding of factors V and Va to phospholipid vesicles by using the technique described in Figure 2A. While several conclusions are consistent with our findings and the raw data are very similar, differences in interpretation are evident. Bloom et al. observed less than quantitative binding of added protein to the membrane. They assumed that unbound protein was in equilibrium and used this to calculate binding constants. No evidence was presented to support this assumption. Control experiments establish that, in our experiment, the unbound protein was not in equilibrium and the failure to observe 100% membrane-binding protein in some preparations was due to other factors.

The number of factor V binding sites per vesicle varied depending on the density of charged phospholipids in the membrane (Table I). At 5 and 10% PS, binding was probably limited by the availability of phosphatidylserine. Given that approximately two-thirds of the phosphatidylserine molecules were on the outside of the vesicles and were used for factor V binding, the number of binding sites per vesicle of 5 and 10% phosphatidylserine suggested 25–30 acidic phospholipids per factor V binding site. This is similar to the number of residues involved in myelin basic protein–membrane association (Boggs et al., 1981).

This large requirement for acidic phospholipids suggested an ionic interaction between factor V and the membrane. The dissociation rate studies strongly supported this conclusion. The dissociation rate greatly increased with ionic strength (Figure 6), and the entire complex could be dissociated by very high salt. Calcium ion, which binds to the membrane and neutralizes charge, also increased the dissociation rates. The dissociation rate was also sensitive to the charge density in the membrane (Table I, compare 10 and 20% PS). The pH effects

suggested that deprotonation of lysine residues may increase the dissociation rate. In light of these findings, an unexpected observation was the lack of an ionic strength effect on the association process. Figure 3 shows that a 10-fold change in ionic strength had a very small affect on the association rate constant. The charge density in the membrane also had little effect on the association rate; the collisional efficiency was approximately proportional to the number of binding sites on the membrane (Table I). These findings may suggest two types of protein–membrane interactions. Initial membrane contact may involve nonionic forces. The complex may be stabilized by subsequent clustering of acidic phospholipids around the protein. The latter process would be inhibited by ionic strength, and the dissociation rate would be altered. Other possible explanations for these different characteristics may be found with future study.

Enoch et al. (1977) and Leto et al. (1980) have shown that cytochrome b_5 and b_5 reductase dissociate from membrane vesicles. The time required was nearly 100-fold longer than for factor V–membrane dissociation. The rate of factor V–membrane dissociation therefore lies midway between that of these intrinsic membrane proteins and that of prothrombin (k_2 of about 4 s^{-1}; Wei et al., 1982). Comparison of association rate constants is not possible due to self-association of intrinsic membrane proteins such as cytochrome b_5 (Spatz & Strittmatter, 1971).

The affinity of factor V for these synthetic membranes (K_D of about 1×10^{-10} M) closely resembles the reported affinity of factor Va for binding sites on the platelet (Tracy et al., 1981). The acidic phospholipid content of these synthetic membranes was selected to resemble relevant physiological membranes. It appears possible that the interaction of factor Va with platelets is due entirely to phospholipids.

Thrombin treatment of factor V converts it to factor Va, the biologically active form. While extended thrombin treatment will inactivate the protein, control studies indicated that our measurements were made with maximally functional protein. Thrombin treatment removed relatively little protein mass from the phospholipid, but it caused a major decrease in the degree to which the protein extended from the membrane surface (Table II). This observation correlates well with the reported change in the hydrodynamic radius of factor V (9.5 nm) upon conversion to factor Va (5.9 nm) (Esmon, 1979).

Thrombin digestion was necessary for substantial interaction with the vitamin K dependent proteins (Figure 7). The results demonstrated that factor X, factor Xa, and prothrombin decreased the rate of factor Va–membrane dissociation. The most likely explanation for these effects is an association between two membrane-bound proteins producing a complex with two membrane association sites. Factor Xa was the most effective in this capacity. This agreed with the very tight binding of factor Xa to platelets (Miletich et al., 1978; Tracy et al., 1981; Dahlbäck & Stenflo, 1978) and phospholipids vesicles containing factor Va (Nesheim et al., 1981). Prothrombin and factor X required much higher concentrations to slow factor Va–membrane dissociation. This presumably reflects a lower binding affinity of prothrombin or factor X for factor Va.

Prothrombin fragment 1 increased rather than decreased the rate of factor Va dissociation from the membrane (Figure 7C). This may be caused by protein competition for acidic phospholipids which is not offset by fragment 1–factor Va interactions on the membrane. Others have reported that the factor Va–prothrombin interaction involves the fragment 2 region of prothrombin (Esmon et al., 1974).

The data obtained in this study can be used to calculate several thermodynamic properties of factor V–membrane interaction. The low activation energy of association with membranes of 20% phosphatidylserine, 6.5 kcal/mol, is expected for a diffusionally controlled reaction. This agrees with the high collisional efficiency observed (Table I). The activation energy for dissociation from these same vesicles was 13 kcal/mol. These activation energies give an enthalpy change for binding of −6.5 kcal/mol. The entropy change estimated from these values and the dissociation constant (Table I) was +24 eu.

The studies presented here reveal that the actual membrane-binding site of factorV is not affected substantially by thrombin treatment and is not dependent on retention of procoagulant factor Va activity. This situation is similar to that of prothrombin where the membrane binding peptide (fragment 1, prothrombin residues 1–156), which no longer contains the enzymatically functional region of the protein, can be obtained separately. It may be possible to obtain a smaller segment of factor V that contains the intact membrane-binding site. This would simplify future characterization of this protein–membrane interaction.

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Resolution of the Two Metal Binding Sites of Human Serum Transferrin by Low-Temperature Excitation of Bound Europium(III)†

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ABSTRACT: Derivatives of apotransferrin have been prepared in which europium replaces iron at either one or both of the two metal ion binding sites. At low temperature (77 K), pH 7.0, two sharp absorption lines are seen by means of laser-induced fluorescence of the bound europium. The one at 579.88 nm (17 245 cm^{-1}) is assigned to the C-terminal region A site, and the other at 579.26 nm (17 263 cm^{-1}) is assigned to the N-terminal region B site. The lifetimes of the excited $^5D_0$ states are 210 ± 20 and 310 ± 30 μs for the A and B sites, respectively. The energy difference between the two peaks is a function of pH, with the splitting decreasing from 0.62 nm (18.5 cm^{-1}) at pH 7.0 to 0.15 nm (4.5 cm^{-1}) at pH 8.0. This spectroscopic inequivalence may be explained by a charge difference of the liganding groups at sites A and B.

Human serum transferrin, M_r 80 000, is an iron transport protein composed of a single polypeptide chain and two metal ion binding sites (Aisen & Listowsky, 1980). Preliminary X-ray studies show the protein to be composed of two homologous domains, each containing a small cleft in which binding may occur (DeLucas et al., 1978). Iron may be substituted by a variety of divalent and trivalent metal ions including the lanthanide Eu(III) (Luk, 1971). Designated A (C-terminal region) and B (N-terminal region), the two sites are very similar but not identical in their metal binding properties. Some of the subtle differences observed by a variety of spectroscopic and chemical techniques are (1) a chelate specificity in which $Fe(NTA)_2$[1] directs iron to the A site at low pH (Harris, 1977) and $Fe(citrate)_4$ directs iron to the B site at neutral pH (Zapolski & Princiotto, 1977), (2) a factor of 5 difference in the iron binding constants at physiological pH ($K_{site\,A} = 5K_{site\,B}$) that increases to a factor of 30 at lower pH (Aisen et al., 1978), (3) spectroscopic inequivalence as detected by VO(II) and Cu(II) EPR (Cannon & Chasteen, 1975; Zweier & Aisen, 1977), and (4) observation of in vivo predominance of iron at site B (Leibman & Aisen, 1979). It was our intent to characterize this difference by a new technique for probing metal binding sites in proteins, Eu(III) excitation spectroscopy.

The Eu(III) excitation technique was introduced by Horrocks & Sudnick (1979a,b) in their study of the Ca^{2+} binding protein thermolysin. Under the conditions of their experiments, Eu(III) replaced Ca(II) in three of the four binding sites, and excitation spectra assignable to two distinct environments were observed. The usefulness of the europium ion spectroscopy arises from a unique electronic transition, $^7F_0 \rightarrow {}^5D_0$. Because both the ground and excited states are inherently nondegenerate, the field due to the ligands can never split the transition. In a homogeneous environment an absorption curve of Lorentzian shape should be observed for the $^7F_0 \rightarrow {}^5D_0$ transition. In fact, the absorption coefficient of this highly forbidden transition is so small ($\epsilon \sim 0.001$ M^{-1} cm^{-1}) that the absorption has never been observed directly but only by fluorescence excitation. Although the degeneracy of the transition is independent of ligand field effects, the energy is not. Sites with different ligand fields will differ slightly in the positions of their resonances. Any structure in the profile is therefore due to heterogeneity of the sites. Thus, by measuring the fluorescence excitation spectrum of transferrin in which Eu(III) has been substituted for Fe(III), we were able to identify and resolve transitions due to the two different sites.

Experimental Procedures

Sample Preparation. Apoprotein samples from Calbiochem Behring were used without further purification after gel electrophoresis showed them to contain no contaminants. Concentrations used were 0.2–1.0 mM. Europium(III) chloride hexahydrate (Aldrich, 99.99% pure) was dissolved in water to 25–32 mM. Small aliquots were added to the protein to achieve the desired saturation.

The buffer used for room temperature spectra contained 10 mM Hepes and 5 mM $NaHCO_3$ at pH 7.0. Low-temperature spectra used a buffer of 100 mM Hepps, 10 mM $NaHCO_3$, and 5% sucrose at pH 7.0–8.0. Hepps was chosen for the low-temperature experiments for two reasons: (1) with a pK_a = 8.00, it has a $\Delta pK_a/\Delta T$ value of −0.007 and is therefore resistant to pH changes upon cooling; (2) Hepps does not bind metals and thus will not interfere with metal complexation to the protein and will not introduce adventitious metals into the solutions. The presence of sucrose in the buffer made the protein more resistant to denaturation upon freezing and thawing.

Dieuropic samples were prepared by adding 1.9[M] equiv (where [M] is the protein concentration) of europium stock to the apoprotein sample. Europium was specifically loaded at the A site of transferrin by first incubating the protein with

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[1] Abbreviations: NTA, nitrilotriacetate; $Eu_{A,B}Tf$, dieuropic transferrin; Eu_AFe_BTf, monoeuropic (site A) monoferric (site B) transferrin; Eu_BFe_ATf, monoeuropic (site B) monoferric (site A) transferrin; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; PMT, photomultiplier tube.

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