Phospholipid-Binding Properties of Bovine Factor V and Factor Va†

James W. Bloom,‡ Michael E. Nesheim,‡ and Kenneth G. Mann*

ABSTRACT: Factor V and factor Va binding to single bilayer phospholipid vesicles was investigated by light-scattering intensity measurements. This technique allows the measurement of free and phospholipid-bound protein concentrations from which equilibrium constants can be obtained. As controls, the Ca^{2+}-dependent phospholipid binding of prothrombin and factor X were also studied. The average values obtained for the dissociation constants (K_d) and lipid to protein ratio at saturation, moles/mole (n), for prothrombin ($K_d = 2.3 \times 10^{-6}$ M, $n = 104$) and factor X ($K_d = 2.5 \times 10^{-6}$ M, $n = 46$) binding to vesicles containing 25% Folch fraction III and 75% phosphatidylcholine in the presence of 2 mM Ca^{2+} were in agreement with those reported in the literature. The average factor V and factor Va values for the dissociation constants and lipid to protein ratio at saturation (moles/mole) were $K_d = 7.2 \times 10^{-8}$ M and $n = 270$ for factor V and $K_d = 4.4 \times 10^{-7}$ M and $n = 76$ for factor Va. In contrast to prothrombin and factor X, factor V and factor Va demonstrated Ca^{2+}-independent lipid binding. In addition, the number of factor V and factor Va molecules bound per vesicle was found to be dependent both on the phosphatidylserine content of the vesicle and the ionic strength of the buffer.

Phospholipid is essential for the maximal rate of conversion of prothrombin to thrombin in the process of blood coagulation, presumably because it provides a surface upon which the substrate (prothrombin), catalyst (factor Xa), and activators [factor V (factor Va) and Ca^{2+}] can form a complex, called prothrombinase, and perform their functions (Mann, 1976; Suttie & Jackson, 1977). Knowledge of the nature of the phospholipid–protein interaction in the prothrombinase complex therefore is essential for the eventual reconstruction of the coagulation process.

Factor Xa and prothrombin have been the most thoroughly studied components of the prothrombinase complex. These proteins bind to membranes containing acidic phospholipids in the presence of calcium ions (Papahadjopoulos & Hanahan, 1964; Bull et al., 1972). Quantitative data on the interaction of prothrombin and factor Xa with phospholipid vesicles have been reported utilizing the technique of 90° light scattering (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977). The use of this physical technique allowed the measurement of free and membrane-bound protein concentrations from which equilibrium constants could be obtained.

Several laboratories (Papahadjopoulos & Hanahan, 1964; Jobin & Esnouf, 1967; Greenquist & Colman, 1975; Subbiah et al., 1976) have investigated the interaction of partially purified factor V (factor Va) with various phospholipid preparations. Added calcium ions are apparently not required for factor V or Va binding to membranes (Papahadjopoulos & Hanahan, 1964; Greenquist & Colman, 1975; Subbiah et al., 1976), but conflicting reports exist as to the necessity of the presence of acidic phospholipids in the membrane (Jobin & Esnouf, 1967; Subbiah et al., 1976). These studies are difficult to interpret for several reasons: the factor V (factor Va) preparations were only partially purified and contained as major components nonfactor V proteins; the lipid dispersions were physically uncharacterized; and the methods of analysis used to study these protein–membrane interactions, primarily gel filtration, produce only qualitative results.

Recently, this laboratory has reported the purification and physical characterization of homogeneous bovine factor V (Nesheim et al., 1979a). In addition, factor V was shown to be an inactive or minimally active "pro-cofactor" which can be activated to factor Va by catalytic amounts of thrombin (Nesheim & Mann, 1979). We have utilized purified factor V, thrombin-activated factor V (factor Va), well-characterized single bilayer phospholipid vesicles, and the technique of 90° light scattering to obtain dissociation constants and occupation numbers for the binding of factor V and factor Va to phospholipid vesicles. In order to determine the nature of the protein–membrane interaction, we also have investigated the effect of acidic phospholipids, calcium ions, and high salt concentrations on the binding parameters.

Materials and Methods

Proteins. Prothrombin and factor X were purified from citrated bovine plasma as previously described (Bajaj & Mann, 1973). Bovine α-thrombin was prepared as described by Lundblad et al. (1975). Electrophoretically homogeneous bovine factor V was purified as described previously (Nesheim et al., 1979a). Factor V activity was measured by the ability to correct the prolonged prothrombin time of factor V deficient plasma as described in detail previously (Nesheim et al., 1979a). Factor Va was prepared by adding thrombin (stock solution 200 NIH units/mL) to a final concentration of 2.0 NIH units/mL and incubating at 37 °C for a minimum of 3 min (Nesheim & Mann, 1979). The mixture was then assayed to determine whether complete activation had occurred; if not, the incubation was extended as necessary. After thrombin treatment, the factor V specific activity was typically 1250 units/absorbance unit based upon a bovine plasma standard. Factor Va solutions were stored on ice and used in the binding studies within 3 h of activation.

Protein concentrations were determined and spectrophotometrically corrected for Rayleigh scattering by using the equation

$$A_{280} = A_{280abs} - 1.70A_{320abs}$$

The molecular weights and extinction coefficients ($E_{280nm}^{1\%,1cm}$) of the proteins were taken as follows: factor V and factor Va (Nesheim et al., 1979a), 330 000 and 9.6,

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respectively; prothrombin (Mann & Elion, 1977), 72 100 and 14.4; and factor X (Jackson et al., 1968; Fujikawa et al., 1972), 55 100 and 12.4. The protein preparations were at least 95% homogeneous as evaluated electrophoretically by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (Weber & Osborn, 1969).

Factor Va has been shown to consist of several component polypeptide chains of varying molecular weights (Nesheim & Mann, 1979; Esmon, 1979) that appear to remain noncovalently associated under native conditions (gel electrophoresis in Tris–borate/$CaCl_2$, pH 8.3) in dilute salt. To determine whether the components remain noncovalently associated under the conditions used in the lipid-binding experiments, we undertook the following studies. Factor Va was gel filtered on 6% agarose in 0.01 M Tris, 0.10 M NaCl, and 0.002 M $CaCl_2$, pH 7.4, and two protein peaks were eluted with the most predominant containing all of the factor Va activity. The protein from the peak containing the factor Va activity was then subjected to sedimentation equilibrium analysis, and a preliminary molecular weight of 240 000 was determined. This result suggested that factor Va had been separated by gel filtration into two components of approximate molecular weights of 240 000 and 90 000. However, when factor Va bound to 25% Folch fraction III vesicles was gel filtered under the same conditions, the entire factor Va molecule appeared to be bound to the lipid vesicle. As a result of these preliminary experiments, we have elected to use a value of 330 000 for the molecular weight of factor Va. The maximum error incurred by using 330 000 rather than 240 000 for the molecular weight of factor Va in the calculation of lipid-binding parameters would be 27%, a value well within experimental error.

In preparation for analysis, the proteins were dialyzed in plastic containers against 0.01 M Tris and 0.10 M NaCl, pH 7.4, for ~2 h at 4 °C. In experiments where ion contaminants might be a problem, the proteins were dialyzed against the same buffer containing Chelex resin (Bio-Rad Laboratories, in the sodium form). Factor V dialyzed in this manner retained its structural integrity as judged by bioassay and $NaDodSO_4$ gel electrophoresis.[1] Factor Va was prepared from dialyzed factor V by treatment with thrombin.

Phospholipid Vesicle Preparation. The phospholipids purchased from Sigma Chemical Co. and the purity estimates given by Sigma for the particular lots used in these experiments are the following: soybean phosphatidylcholine (95%) and bovine brain Folch fraction III (80% phosphatidylserine, 10% phosphatidylethanolamine, 5% cerebrosides, and 5% unidentified).

Single lamellar phospholipid vesicles of homogeneous size were prepared by a modification of the method of Barenholz et al. (1977). The phospholipid vesicles were prepared by first weighing out the appropriate amount of Folch fraction III and dissolving it in the appropriate amount of the chloroform solution of phosphatidylcholine (25 mg total lipid) in a flat-bottom glass vial. The lipid–chloroform solution was dried under nitrogen, redissolved in benzene, lyophilized overnight, and either used immediately or stored at −20 °C. The lyophilized powder was then dispersed in 5 mL of 0.1 M NaCl and direct probe sonicated with a Branson sonifier Model W-350 (continuous sonication, 50% duty cycle, output control setting of 5) for 1.5 min, followed by a 1.0-min cooling period for a total sonication time of 30 min. The sample was maintained in an ice bath and under nitrogen during sonication. Following sonication, the vesicle dispersion was centrifuged for 30 min at 35 000 rpm in a Beckman Model L3-50 ultracentrifuge (SW-50.1 swinging bucket rotor) to remove sonicator probe particles and large miltilameller liposomes. The dispersion was centrifuged again at 40 000 rpm for 3 h and the clear supernatant, region III (Barenholz et al., 1977), removed. The resultant homogeneous vesicle dispersion was stored at 4 °C, and the binding studies were usually completed within 10 h of vesicle preparation. Barenholz et al. (1977) have shown that 100% phosphatidylcholine vesicles prepared by their method have a mean vesicle radius of 105 Å. The phospholipid concentration of the stock solution was based on organic phosphate determined by a protocol developed by the Mayo Clinic Lipids Laboratory for serum phospholipid determination (Gomori, 1942) using a (w/w) conversion factor of 25 (phospholipid/P) for phosphatidylserine and phosphatidylcholine. In all cases, phospholipid composition is expressed as the molar ratio of phospholipids present before sonication and centrifugation. Where a vesicle composition is given as a percentage of Folch fraction III, the remainder of the phospholipid is phosphatidylcholine.

Light-Scattering Measurements. Relative 90° light-scattering measurements were made in a Perkin-Elmer Model MPF-44A fluorescence spectrophotometer at room temperature (25 °C). Excitation and emission wavelengths were both set at 320 nm. The sample volume was 1.5 mL and the final phospholipid vesicle concentration varied between 7 and 30 μg/mL. Protein samples were added to the cuvette, and the light scattering from the protein–phospholipid complex was compared to the scatter from phospholipid alone after correction for volume changes. The light-scattering data were analyzed by the method developed by Nelsestuen & Lim (1977). The value of the change in refractive index with concentration ($\partial n/\partial c$) for prothrombin and factor X was 0.192, while the value used for phospholipid was 0.172 (Nelsestuen & Lim, 1977). The refractive index for factor V and factor Va was assumed to be 0.192. This appears to be a reasonable assumption since the amino acid and carbohydrate compositions of factor V and factor Va are not appreciably different from those of prothrombin and factor X (Nesheim et al., 1979a). A 10% variance in the refractive index would lead to the same variance in the calculated K_d, a value well within experimental error.

The measurement of protein–vesicle binding by light scattering is dependent upon a system which does not undergo secondary aggregation of the protein–vesicle complex. This secondary aggregation would cause an overestimate of the amount of protein bound, and the estimated dissociation constant would be low. For this reason, the experimental conditions of calcium ion concentration and vesicle lipid composition were chosen so as to avoid vesicle–vesicle aggregation (Papahadjopoulos et al., 1976; Nelsestuen & Lim, 1977). The prothrombin- and factor X–vesicle interactions were reversible as shown by the addition of ethylenediaminetetraacetic acid (EDTA) to the complex, and thus prothrombin or factor X induced vesicle–vesicle aggregation was not a problem (Lim et al., 1977).

We were able to demonstrate by 90° light-scattering experiments that the 25% Folch fraction III vesicles, at the concentrations used in the binding experiments, could be induced to associate (as indicated by increased light scattering) at Ca^{2+} concentrations greater than 10 mM. The scattering increase was instantaneous upon addition of Ca^{2+} ions and was completely reversible upon the addition of EDTA. If allowed

[1] In this instance the protein was dialyzed vs. Chelex in buffer at a concentration (w/v) of 1 g/L for 2 h. Treatment of factor V/Va with large amounts of Chelex for prolonged intervals will lead to activity loss due to intrinsic calcium removal.

Table I: Phospholipid-Binding Properties of Prothrombin and Factor X

protein	$[Ca^{2+}]$ (mM)	total lipid concn[a] (μg/mL)	K_d ($\times 10^{-6}$) (M)	n[b]
prothrombin	2	26.6	2.31	104
	1	66.0	5.43	132
	1	80.0	0.90[c]	71[c]
factor X	2	13.6	2.62	40
	2	26.6	2.45	48
	2	30.2	2.35	51
	1	80.0	0.40[c]	91[c]

[a] 25% Folch fraction III. [b] Lipid to protein ratio at saturation (moles/mole). [c] Calculated from the relative molecular weight data (Figure 1) presented in the paper by Nelsestuen & Lim (1977).

to stand in the presence of Ca^{2+} concentrations greater than 10 mM for a period of at least 10 min, however, a slow increase in the scattering of the vesicles was observed, suggesting additional formation of aggregates (Suurkuusk et al., 1976; Mysels, 1959).

Light-scattering experiments were also carried out in which lipid vesicle samples were added to a cuvette containing protein. The binding process was saturable, indicating that the protein did not induce vesicle–vesicle aggregation. Data obtained in this manner, however, cannot be easily analyzed by the method developed by Nelsestuen & Lim (1977).

Results

Prothrombin and Factor X Phospholipid Binding. The binding of prothrombin and factor X to phospholipid vesicles was studied first, primarily for use as controls. Equilibrium dissociation constants (K_d) and the lipid to protein ratio at saturation, moles/mole (n), were calculated from double-reciprocal plots of the analyzed scattering data (Nelsestuen & Lim, 1977) and are shown in Table I at different lipid and calcium concentrations. The prothrombin-binding experiments were done with stock solutions of protein containing 2 mM Ca^{2+} so that the prothrombin was in its phospholipid-binding conformation (Nelsestuen et al., 1976; Bloom & Mann, 1978). At 2 mM Ca^{2+} and with 25% Folch fraction III vesicles, both prothrombin and factor X have essentially the same K_d values (on the order of 10^{-6} M), but the n for prothrombin is approximately twice the factor X value. The equilibrium constants listed in Table I are compared with the values we have calculated from the relative molecular weight vs. protein/phospholipid graph at 1 mM Ca^{2+} for 25% phosphatidylserine vesicles (Figure 1) shown in Nelsestuen & Lim (1977). The equilibrium constants determined from the scattering experiments done in the two laboratories appear to agree very well. The differences in the values can probably be attributed primarily to differences in the phospholipid compositions of the vesicles and Ca^{2+} concentrations.

Nelsestuen and Broderius (1977) have determined that the membrane-binding characteristics of factor X are similar if not identical to those for factor Xa. For purposes of discussion then, factor X and factor Xa will be assumed to have identical equilibrium binding parameters.

Factor V and Factor Va Lipid Binding. The results of a typical experiment of factor Va binding to 25% Folch fraction III vesicles are shown in Figures 1 and 2. The experimental scattering data are shown in Figure 1, while the relative molecular weight analysis is shown in Figure 2 (a double-reciprocal plot is shown in the inset). The K_d and n values calculated for this factor Va experiment are 5.42×10^{-7} M and 62, respectively.

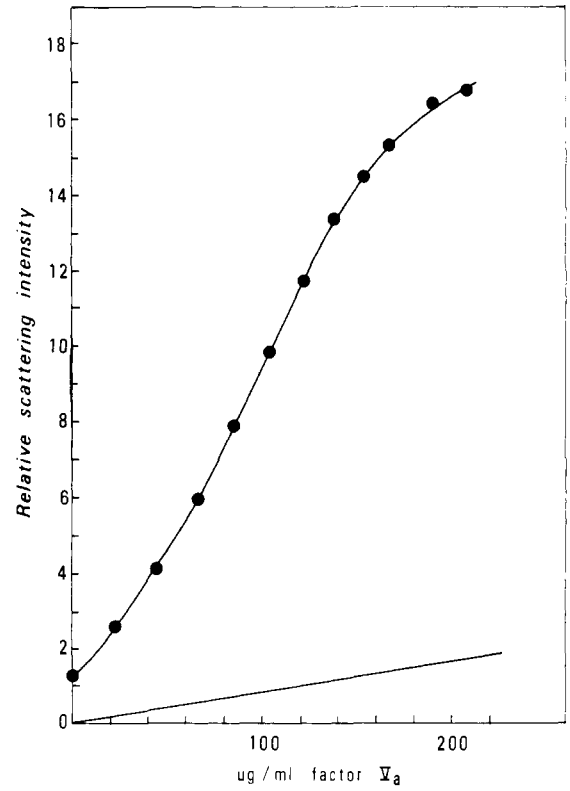

FIGURE 1: Light-scattering data for the binding of factor Va to 25% Folch fraction III vesicles (26.9 μg/mL total lipid concentration). The straight line without data points is the scattering from factor Va alone.

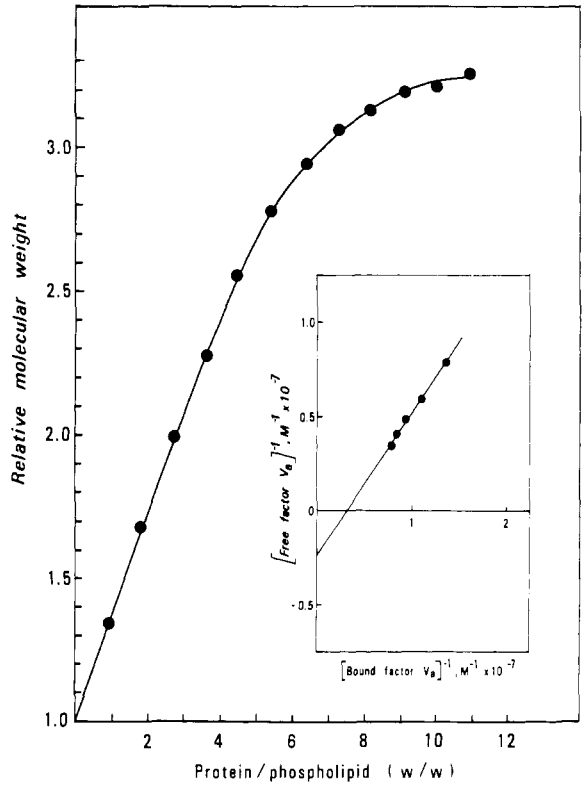

FIGURE 2: Relative molecular weight determination of the light-scattering data shown in Figure 1. The light scattering from the phospholipid vesicles alone has been assigned a value of 1.0, and the values plotted have been corrected for light scattering from unbound protein. The inset shows a double-reciprocal plot of the data. These figures do not include corrections made for volume changes. The K_d and n values calculated from this experiment, including corrections for volume changes, were 5.42×10^{-7} M and 62, respectively.

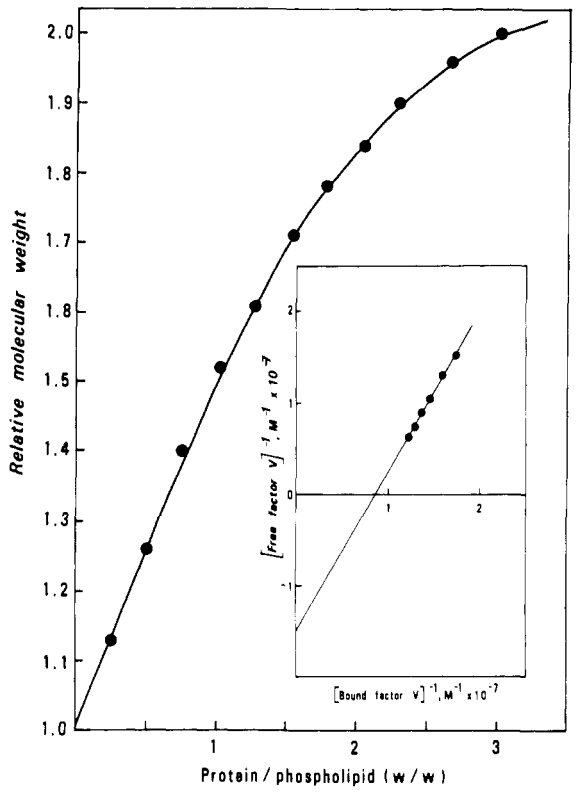

FIGURE 3: Relative molecular weight determination by light scattering for factor V. The light scattering from the phospholipid vesicles alone (25% Folch fraction III, 26.9 μg/mL total lipid concentration) has been assigned a value of 1.0, and the values plotted have been corrected for light scattering from unbound protein but not for volume changes. The inset shows a double-reciprocal plot of the data. The K_d and n values calculated from this experiment, including corrections for volume changes, were 9.84×10^{-8} M and 213, respectively.

The molecular weight of factor Va is subject to uncertainty and is difficult to define. On the basis of preliminary sedimentation equilibrium experiments of factor Va resolved from peptide material not noncovalently associated at the pH and ionic strength used in the light-scattering experiment, a noncovalently associated product of ~240 000 daltons is observed. Results published by Esmon (1979) as well as results published by our laboratory (Nesheim & Mann, 1979) indicate that the minimum active species for factor Va is even smaller than this (M_r 167 000). The present experiments were performed by using factor Va without fractionation of the noncovalently associated species, the minimum molecular weight of which is ~240 000. Since it is still not clear that the activation peptide does not also bind to lipid, we have calculated our data based upon a molecular weight of 330 000 for factor Va. If a value of 240 000 is used for the molecular weight of factor Va, however, the calculated K_d (7.45×10^{-7} M) and n (45) values for the binding of factor Va to 25% Folch fraction III vesicles are within the experimental error inherent in the light-scattering experiments.

A factor V binding experiment using the same phospholipid preparation as above is shown in Figure 3. The K_d and n values calculated for this factor V experiment are 9.84×10^{-8} M and 213, respectively. These results indicate that factor V binds to these 25% Folch fraction III vesicles with 10 times the affinity of factor Va, but ~3 times the number of factor Va molecules are bound per vesicle than factor V. Both factor V and factor Va bind to 25% Folch fraction III vesicles with higher affinities than prothrombin or factor X.

Calcium Dependence of Factor V and Factor Va Phospholipid Binding. To investigate the potential role of Ca^{2+} in factor V and factor Va phospholipid binding, we undertook the following experiments. Factor V was dialyzed against buffer containing Chelex resin in order to remove any contaminant Ca^{2+}. Chelex was chosen as a chelating agent because it had little or no effect on factor V activity.[1] EDTA on the other hand eliminated factor V procoagulant activity. The K_d and n values for factor V binding to 25% Folch fraction III vesicles were determined to be 4.62×10^{-8} M and 327, respecitvely. The experiment was then repeated but this time in the presence of 2 mM Ca^{2+} with the following results: $K_d = 4.54 \times 10^{-8}$ M and $n = 398$. Factor Va binding in the presence and absence of 2 mM Ca^{2+} was also investigated and found to have K_d and n values identical within experimental error: in the absence of exogenous Ca^{2+}, $K_d = 5.42 \times 10^{-7}$ M and $n = 62$; in the presence of 2 mM Ca^{2+}, $K_d = 4.22 \times 10^{-7}$ M and $n = 84$. Thus, the parameters for both factor V and factor Va binding to 25% Folch fraction III vesicles were unaltered whether Ca^{2+} ions were present or absent from the medium.

In case Chelex had not completely removed contaminant Ca^{2+}, factor V binding to 25% Folch fraction III vesicles was studied in the presence of EDTA. A 0.7 mg/mL solution of factor V was made 5 mM in EDTA and allowed to stand at room temperature (25 °C) for 4 h. The sample was then assayed for activity not only before but also after thrombin activation. After EDTA treatment the activity measured prior to activation by thrombin, which is assumed to be the result of endogenous activated cofactor (Nesheim et al., 1979a), had decreased by 50%. The activity observed after thrombin activation, which is assumed to be a measure of total potential activity in the form of pro-cofactor (Nesheim et al., 1979a), however, had decreased by 95% of the starting value and control. The K_d and n values for the binding of the EDTA-treated factor V to 25% Folch fraction III vesicles were 7.70×10^{-8} M and 271, respectively. The 24 μg/mL phospholipid solution to which aliquots of the EDTA-treated factor V were added also contained 5 mM EDTA. The untreated factor V control gave K_d and n values of 2.59×10^{-8} M and 343, respecitvely. Thus, EDTA-treated factor V binds 25% Folch fraction III vesicles but with a slightly lower affinity than the untreated factor V. This decrease in affinity appears to be correlated with a loss of activity of the protein. The binding of factor V and factor Va to 25% Folch fraction III vesicles then does not appear to be dependent on Ca^{2+}.

Effect of High NaCl Concentrations on Factor V and Va Lipid Binding. Proteins associated with membranes are often referred to as having an intrinsic or extrinsic relationship with the membrane. Intrinsic proteins are believed to bind to the membrane lipid through hydrophobic interactions and require detergents for membrane release. Extrinsic proteins on the other hand are thought to bind to membrane lipids through ionic forces and require chelating agents or high salt concentrations for release. The Ca^{2+}-independent lipid binding of factor V and factor Va indicates that the mechanism of binding to lipid for the proteins is different from the Ca^{2+} bridging mechanism of prothrombin and factor X (Lim et al., 1977; Bloom & Mann, 1978). To determine whether factor V and factor Va bind to lipid through some other type of electrostatic interaction, we examined the effect of high NaCl concentrations on lipid binding. In these experiments a 15-μL aliquot of a concentrated stock solution of 25% Folch fraction III vesicles in 0.1 M NaCl was added to the cuvette which contained 1.5 mL of 0.02 M Tris, pH 7.4, plus the appropriate NaCl concentrations. The K_d and n values for factor V and factor Va binding to 25% Folch fraction III vesicles at different

Table II: Effect of Various NaCl Concentrations on Factor V and Factor Va Lipid[a] Binding

protein	[NaCl] (M)	K_d (M)	n^b
factor V	0.15	4.62×10^{-8}	327
	0.5	4.82×10^{-8}	929
	1.0	9.46×10^{-8}	2305
factor Va[c]	0.15	5.42×10^{-7}	62
	0.5	9.01×10^{-7}	93
	1.0	1.83×10^{-7}	2008

[a] 25% Folch fraction III. [b] Lipid to protein ratio at saturation (moles/mole). [c] Calculations based on a molecular weight for factor Va of 330 000.

NaCl concentrations were determined and are presented in Table II. With increasing NaCl concentrations the factor V and factor Va dissociation constants remained relatively unchanged while the lipid to protein ratio increased.

High salt concentrations cause a decrease in the scattering of the vesicles alone, suggesting vesicle shrinkage. At 0.5 M NaCl the scattering intensity of a 27 μg/mL solution of 20% phosphatidylserine vesicles decreased by 9% compared to the value at 0.15 M NaCl, while at 1.0 M NaCl the scattering intensity is 13% less than the value at 0.15 M. The increase in the number of lipids bound per factor V or factor Va molecule at high salt concentrations therefore most likely is representative of a change in the physical state of the vesicles at high ionic strength. Whether this observation is the result of a change in the radius of curvature of the vesicles, an alteration of the microviscosity of the membrane, or a redistribution of the phosphatidylserine residues to the interior surface of the membrane is not known. The finding that the factor V and factor Va dissociation constants remain essentially unchanged by increasing NaCl concentrations indicates that the binding of these proteins to 25% Folch fraction III vesicles is most likely not electrostatic in nature.

Lim et al. (1977) have shown that prothrombin and factor X do not induce vesicle–vesicle aggregation. Such a secondary aggregation will cause an overestimate of the amount of protein bound, and the estimated dissociation constants will be low (Nelsestuen & Lim, 1977). The following observations suggest that factor V and factor Va also do not cause vesicle–vesicle aggregation. (1) Sedimentation equilibrium experiments performed in this laboratory show that at the protein concentrations used in the light-scattering experiments factor V and factor Va do not aggregate. (2) The finding that the dissociation constants for factor V and factor Va binding to 25% Folch fraction III vesicles are independent of high salt concentration also argues against secondary aggregation of the form vesicle–protein–protein–vesicle. (3) Factor V and factor Va binding to lipid vesicles is a saturable process. Protein-catalyzed vesicle–vesicle aggregation would not be expected to be saturable. (4) The slow time-dependent increase in light scattering intensity which occurred during Ca^{2+}-induced secondary aggregation is not observed in the protein-binding experiments. These observations strongly suggest that, as in the case of prothrombin and factor X, factor V and factor Va do not induce vesicle–vesicle aggregation.

Factor V and Factor Va Binding as a Function of Phosphatidylserine Content. It has been demonstrated that prothrombin and factor X membrane binding requires the presence of an acidic phospholipid; phosphatidylserine was found to be the most effective acidic phospholipid in promoting the interaction (Nelsestuen & Broderius, 1977). In order to determine whether the factor V and factor Va membrane interaction is also dependent on the presence of acidic phospholipid, we carried out binding experiments with vesicles of varying Folch fraction III content.

Table III: Factor V and Factor Va Binding to Vesicles of Varying Folch Fraction III (Ff III)[a] Content

fraction Ff III (%)	total lipid concn (μg/mL)	factor V		factor Va[b]	
		K_d (M)	n^c	K_d (M)	n^c
0.0		no binding		no binding	
12.5	28.8	1.11×10^{-7}	470	4.83×10^{-7}	117
25.0	24.1	4.62×10^{-8}	327	3.37×10^{-7}	91
	26.9	9.84×10^{-8}	213	5.42×10^{-7}	62
40.0	8.4	2.34×10^{-8}	315	8.99×10^{-8}	120
	25.0	6.21×10^{-8}	240	ND	
50.0	9.0	ND[d]		5.10×10^{-8}	88
	26.7	8.20×10^{-8}	84	ND	
75.0	6.9	ND		8.50×10^{-8}	66
	20.5	1.14×10^{-8}	165	ND	
	20.5	1.42×10^{-8}	129	ND	

[a] 80% phosphatidylserine, 10% phosphatidylethanolamine, 5% cerebrosides, and 5% unidentified. [b] Calculations based on a molecular weight of 330 000. [c] Lipid to protein ratio at saturation (moles/mole). [d] ND, not determined.

The experimental results of varying Folch fraction III content from 0 to 75% are presented in Table III. Factor V and factor Va do not bind to 100% phosphatidylcholine vesicles. It appears, moreover, that the affinity of factor V for vesicles of decreasing phosphatidylserine content falls off much more rapidly than the affinity of factor Va. At values of greater than 40% Folch fraction III, however, the affinities of factor V and factor Va are nearly identical.

Nelsestuen & Broderius (1977) found that below ~20% phosphatidylserine content the factor X and prothrombin binding capacities were directly proportional to phosphatidylserine content, and the factor limiting protein binding was assumed to be the availability of phosphatidylserine. The data presented in Table III suggest that the binding capacity of factor V and factor Va is directly proportional to Folch fraction III content in the region below ~25% Folch fraction III (or ~20% phosphatidylserine). Above 25% Folch fraction III the amount of factor V or factor Va bound per mole of lipid increases in a relatively nonlinear fashion. The results at high phosphatidylserine concentrations are also analogous to the binding data for prothrombin and factor X (Nelsestuen & Lim, 1977). It has been suggested that the limiting factor in prothrombin and factor X binding is the packing of protein on the membrane surface (Lim et al., 1977). Protein packing probably also limits factor V and factor Va lipid binding.

The results of these experiments suggest that the binding of factor V and factor Va to membranes is dependent on the presence of acidic phospholipids such as phosphatidylserine. These findings along with the results of the high salt and Ca^{2+} experiments indicate that factor V and factor Va bind to phospholipid vesicles by a Ca^{2+}-independent, phosphatidylserine residue dependent, nonelectrostatic interaction.

Discussion

Previous studies (Papahadjopoulos & Hanahan, 1964; Jobin & Esnouf, 1967; Greenquist & Colman, 1975; Subbiah et al., 1976) of factor V and factor Va binding to lipid made use of impure preparations of factor V and methods of analysis which yield only qualitative results. The present studies (which utilize a method of analysis that yields quantitative equilibrium binding data) were made possible by the recent development of a method to prepare factor V of high purity (Nesheim et al., 1979a). It has further been shown in our laboratory (Nesheim & Mann, 1979) that factor V is a pro-cofactor which is activated to factor Va by catalytic amounts of

thrombin through well-defined proteolytic cleavages to produce an 80-fold increase in activity.

Factor V was shown, in this report, to bind to phospholipid vesicles with about 10 times the affinity of factor Va and 100 times the affinity of either prothrombin or factor X.

The rates of activation of prothrombin have been measured in our laboratory by using the thrombin substrate dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (Nesheim et al., 1979b; M. E. Nesheim, J. B. Taswell, and K. G. Mann, unpublished experiments). The proteins used in these studies were prepared to homogeneity, and the lipid was the same 25% Folch fraction III vesicle preparation as used in these light-scattering experiments. Our results, obtained from the prothrombin activation experiments, can be interpreted as being reflective of factor Va binding to the lipid vesicles with an apparent K_d of 7.3×10^{-7} M. These values are in very good agreement with the K_d obtained by light scattering (4.4×10^{-7} M).

In contrast to the other prothrombinase proteins, prothrombin and factor Xa, factor V and factor Va are not displaced from the lipid surface by chelators. However, as in the case of prothrombin and factor Xa, factor V and factor Va require the presence of an acidic phospholipid such as phosphatidylserine for lipid binding. The nature of this interaction, however, differs between prothrombin and factor Xa, which are vitamin K dependent proteins, and factor V and factor Va, which are not. Calcium ions are believed to act as bridges between the factor Xa and prothrombin lipid-binding sites, believed to be γ-carboxyglutamic acid residues, and phosphatidylserine molecules on the lipid surface (Lim et al., 1977; Bloom & Mann, 1978). Factor V and factor Va, on the other hand, which do not contain γ-carboxyglutamic acid residues (unpublished observations), appear to bind to phosphatidylserine-containing phospholipid vesicles through nonelectrostatic interactions. That the factor V and factor Va lipid interaction is nonelectrostatic in nature is perhaps not too unexpected since hydrophobic chromatography is an important step in the preparation of factor V (Nesheim et al., 1979a). The apparent direct dependence (below 20% phosphatidylserine) of the protein–lipid nonelectrostatic interaction on the presence of an acidic phospholipid is, however, not easy to interpret. Differences in the fatty acid chains or the fact that the polar head group of phosphatidylserine is smaller than the choline head group may be factors. Also, it has been observed that in the presence of calcium, phosphatidylserine residues form aggregates with hydrophobic surfaces (Ohnishi Ito, 1974) in phosphatidylserine–phosphatidylcholine membranes. Factor V and factor Va, however, bind equally well to phosphatidylserine–phosphatidylcholine vesicles in both the presence and absence of calcium ions.

These results support the generally held hypothesis that prothrombin activation occurs on a membranelike surface. The effect of calcium ions on membranes composed of mixtures of phosphatidylserine and phosphatidylcholine has been studied (Ohnishi & Ito, 1974). Membranes with low phosphatidylserine content were found to contain aggregates of phosphatidylserine residues bridged by calcium ions which were distributed like islands in a sea of phosphatidylcholine molecules. The fact that the proteins comprising the prothrombinase complex show phosphatidylserine-dependent lipid binding suggests that this calcium-dependent phosphatidylserine aggregation on the lipid surface may act as a means of bringing the proteins into direct physical contact.

Subsequent to vascular injury, aggregated platelets are believed to provide a catalytic surface for localized activation of the prothrombinase complex. Lipid preparations in in vitro studies of prothrombin activation are therefore believed to substitute for platelets in vivo. A dissociation constant of 10^{-10} M (Dahlback & Stenflo, 1978) has been reported for the binding of factor Xa to platelets, while studies in our laboratory (Tracy et al., 1979; P. B. Tracy, J. M. Peterson, M. E. Nesheim, F. C. McDuffie, and K. G. Mann, unpublished experiments) yielded a dissociation constant of 10^{-10} M for the binding of factor Va to platelets. Factor Xa and factor Va therefore appear to bind to platelets with much higher affinities than they do to Folch fraction III–phosphatidylcholine vesicles. This suggests that these coagulation factors do not bind to platelets through simple protein–lipid interactions, and indeed it has been proposed that the factor Xa receptor on platelets is factor V or factor Va (Miletich et al., 1977, 1978). These findings indicate that, in addition to protein–lipid binding, protein–receptor interactions may play a significant role in the organization of prothrombin, factor Xa, and factor Va on the surface of lipid vesicles or platelets.

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Interaction of Divalent Cations and Polymyxin B with Lipopolysaccharide†

Melvin Schindler and M. J. Osborn*

ABSTRACT: Cation binding to the lipopolysaccharide of *Salmonella typhimurium* was investigated by equilibrium dialysis and fluorometric titration of lipopolysaccharides derivatized with dimethylaminonaphthalenesulfonyl chloride (dansyl chloride). In the presence of Tris buffer (50 mM), addition of Ca^{2+} or Mg^{2+} resulted in a blue shift in the emission maximum and an increase in the relative fluorescence of dansylated lipopolysaccharides obtained from the rough mutants G30 (galactose deficient, R_c chemotype) and G30A (heptose deficient, R_e chemotype). These effects were completely reversed by EDTA. Fluorometric titrations revealed two types of binding sites with markedly different affinities for divalent cations in both lipopolysaccharides. The first class, of relatively low affinity, gave K_d values of 0.2 and 1–2 mM with G30A and G30, respectively, and was attributed to pyrophosphoryl and/or phosphodiester groups of the 3-deoxy-*manno*-octulosonate (KDO)–lipid A region of the molecule. The second class, of higher affinity, yielded K_d values for Ca^{2+} and Mg^{2+} of 6 and 15 μM, respectively, with both lipopolysaccharides. Titrations with polymyxin B showed only high-affinity binding with K_d values of 0.3 μM for G30A and 0.5 μM for G30. The nature of the high-affinity Ca^{2+} and Mg^{2+} binding site was further investigated by equilibrium dialysis of lipopolysaccharides and related products with $^{45}Ca^{2+}$. All lipopolysaccharides tested (wild type, G30, and G30A) bound 1 mol of Ca^{2+} per mol of lipopolysaccharide monomer with a K_d of 12–13 μM. In contrast, lipid A (obtained by mild acid hydrolysis of lipopolysaccharide) and an incomplete biosynthetic precursor of lipid A lacking 3-deoxy-D-*manno*-octulosonate yielded K_d values of 100 and 56 μM and a reduction in the stoichiometry of binding to 0.5 and 0.3 mol of Ca^{2+} per mol, respectively. The results suggest that the branched 3-deoxy-D-*manno*-octulosonate trisaccharide unit of lipopolysaccharide may afford a specific high-affinity site for interaction with divalent cations required for assembly and maintenance of the normal structural organization of the outer membrane.

It now appears well established that the lipopolysaccharides located in the external leaflet of the outer membrane of gram-negative enteric bacteria and their interactions with cations are important for the barrier function of the membrane (Leive, 1974; Sanderson et al., 1974; Galanos et al., 1977). Lipopolysaccharide contains a number of potential cation binding sites, and electrodialysis is necessary to remove tightly bound inorganic cations and polyamines from isolated lipopolysaccharide (Galanos & Luderitz, 1975). Both lipid A and the backbone region of the polysaccharide contain phosphate, and in addition a cluster of three carboxyl groups is afforded by the unique branched KDO[1] trisaccharide unit which links polysaccharide to lipid A (Figure 1). It has been postulated by Leive (1974) and others (van Alphen et al., 1978) that divalent cations form ionic bridges between neighboring lipopolysaccharide phosphate groups, stabilizing the outer-membrane structure. However, it is also possible that the KDO residues can form a cage to coordinate Ca^{2+}, Mg^{2+}, or other cations, as has been established for sialic acid. Jaques et al. (1977) have measured 1:1 associations of Ca^{2+} to sialic acid, while Sillerud et al. (1978) have found that the single sialic acid in GM_1 ganglioside was intimately involved through its carboxyl and glycero side chain with cation binding. In fact, other sugars linked to sialic acid also contributed to the metal cation coordination sphere, thus raising the affinity constant beyond that of sialic acid alone.

To define more clearly the nature of the metal binding sites of lipopolysaccharide, we have employed fluorescence analysis of dansylated derivatives of various lipopolysaccharides. In addition, equilibrium dialysis measurements were performed with $^{45}Ca^{2+}$ to provide information about the stoichiometry of binding. The results indicate that the lipopolysaccharides contain a high-affinity binding site for Ca^{2+} and Mg^{2+} which appears to be formed by the three KDO residues of the molecule.

Materials and Methods

Materials

Bacterial Strains and Media. Salmonella typhimurium LT2 and strain G30, a mutant of *S. typhimurium* LT2 lacking UDPgalactose-4-epimerase, have been described (Osborn et al., 1962). Strain G30A is a mutant of G30 which produces a heptoseless lipopolysaccharide (Osborn et al., 1972).

† From the Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06032. *Received June 11, 1979.* This investigation was supported in part by U.S. Public Health Service Grant AI08650.

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[1] Abbreviations used: KDO, 3-deoxy-D-*manno*-octulosonic acid; DNS, dimethylaminonaphthalenesulfonyl chloride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; AraN, 4-amino-4-deoxy-L-arabinose.

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