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Factor Va-Factor Xa Interaction. Effects of Phospholipid Vesicles of Varying Composition†

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ABSTRACT: The interaction between factor Xa and factor Va was investigated both in solution and in the presence of phospholipid vesicles with varying contents of phosphatidylserine. The binding parameters were inferred from the kinetics of prothrombin activation. Factor Xa and factor Va form in solution an equimolar complex with a dissociation constant of 3.3×10^{-9} M. Phospholipid vesicles promote the formation of the factor Xa-Va complex. The K_d of complex formation is dependent on both the phospholipid concentration and the composition of the phospholipid vesicle. For the interaction between factor Xa and factor Va in the presence of phospholipid vesicles containing 40 mol % dioleoylphosphatidylserine (DOPS) and 60 mol % dioleoylphosphatidylcholine (DOPC), the K_d increases linearly with increasing phospholipid concentration. In the presence of 10 μM phospholipid (DOPS/DOPC, 40/60 mol/mol) $K_d = 3 \times 10^{-11}$ M. When the mole percentage of DOPS in the phospholipid vesicles is lowered from 20 to 5 mol %, there is a gradual increase of the K_d. In the presence of 10 μM phospholipid vesicles containing 5 mol % DOPS and 95 mol % DOPC $K_d = 2.8 \times 10^{-10}$ M. The K_d measured in the presence of phospholipid vesicles containing 5 mol % DOPS and 95 mol % DOPC is independent of the phospholipid concentration. Two models are discussed that can quantitatively explain the effect of phospholipid vesicles on the complex formation between factor Xa and factor Va. Studies on the effect of the polypeptides with M_r 80 000 and M_r 94 000 of which factor Va is composed on the K_d of the factor Xa-Va complex suggest that factor Xa binding to factor Va requires a Ca^{2+}-mediated interaction between the two polypeptides.

The activation of blood coagulation factor II (prothrombin)[1] into thrombin, catalyzed by the serine protease factor Xa, requires at physiological conditions the presence of the nonenzymatic components phospholipid, Ca^{2+} ions, and factor Va. It has been shown that these so-called accessory components augment the rate of prothrombin activation and that a prothrombinase complex composed of factor Xa, negatively charged phospholipid, Ca^{2+}, and factor Va is the most efficient in catalyzing prothrombin activation (Suttie & Jackson, 1977).

Kinetic studies on prothrombin activation by prothrombinase complexes of different composition (factor Xa in either the absence or presence of phospholipid, Ca^{2+}, and/or factor Va) reveal that phospholipids cause a profound decrease of the K_m for prothrombin, while factor Va causes a drastic increase of the V_{max} of thrombin formation (Rosing et al., 1980). The changes of the kinetic parameters caused by the accessory components are consistent with a model in which phospholipid provides a surface upon which factor Xa, factor Va, and prothrombin interact at increased local concentrations and factor Va changes the catalytic capacity of factor Xa.

Another important function of factor Va in haemostasis was discovered by Miletich et al. (1978). Their experiments suggest that factor Va is part of a highly specific factor Xa binding site on human blood platelets. The same tight binding site for factor Xa is also present at the surface of bovine blood platelets (Dahlbäck & Stenflo, 1978). Recently, Tracy et al. (1981) demonstrated that factor Xa and factor Va interact stoichiometrically at the platelet surface and that factor Xa binds to platelet-bound factor Va with an apparent dissociation constant of 6×10^{-10} M. Similar binding parameters were obtained in a phospholipid model system (Nesheim et al., 1979b).

The precise mode of action of phospholipid in promoting the interaction between factor Xa and factor Va is not known. In fact, it is not unequivocally demonstrated that factor Xa-Va complex formation at the platelet surface is the result of protein-phospholipid interaction per se. While Bevers et al.

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[1] The nomenclature of the blood coagulation factors used is that recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and Zymogen Intermediates.

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(1982) reported that triggering of platelets with collagen plus thrombin results in the exposure of phosphatidylserine molecules at the platelet outer surface with a concomitant increase in prothrombinase activity, Kane et al. (1980) and Tracy et al. (1981) observed that platelet activation is not required for the interaction of factor Xa with factor Va at the platelet surface.

The purpose of the experiments described in this paper is to assess the contribution of protein–protein and protein–phospholipid interactions to the specificity and high affinity of the binding of factor Xa to phospholipid-bound factor Va. Therefore, we studied the interaction of factor Xa with factor Va in the absence and in the presence of phospholipid vesicles with a varying content of phosphatidylserine. In a first attempt to reveal the structural requirements of the factor Va molecule for binding factor Xa, we also studied the interaction between factor Xa and isolated subunits with molecular weights of 80 000 and 94 000 of which factor Va is composed (Esmon, 1979).

Materials and Methods

S 2238[2] was purchased from AB Kabi Diagnostica. Benzamidine and DFP were obtained from Sigma. All reagents used were of the highest grade commercially available.

Proteins. Bovine factors X_1 and X_2 were prepared according to Fujikawa et al. (1972a). RVV-X was purified from the crude venom as described by Schiffman et al. (1969). Bovine factor Xa was prepared from bovine factor X_1 with RVV-X according to the method of Fujikawa et al. (1972b).

Bovine prothrombin and thrombin were purified as described earlier (Rosing et al., 1980). Prethrombin 1 was purified according to the method of Owen et al. (1974). Decarboxyprothrombin was a kind gift of Dr. Vermeer.

Bovine factor V was purified by a modification of the method of Esmon (1979). Bovine blood was obtained by venipuncture of cows treated with phenprocoumon (average levels of the factors II and X were 20% and 5%, respectively). The blood was collected in 1-L plastic bottles containing 100 mL of 100 mM sodium oxalate, 100 mM benzamidine, 20 000 units of heparin, and 0.2 mg/mL of soybean trypsin inhibitor. Plasma (12 L) was obtained by centrifugation of the blood at 4 °C for 25 min at 2500 rpm in a MSE Mistral-6L centrifuge. $BaSO_4$ (50 mg/mL) was added to the plasma, and the suspension was stirred for 15 min at 4 °C. The plasma supernatant after $BaSO_4$ adsorption was adsorbed to QAE-Sephadex (1 L of swollen gel equilibrated in 10 mM Tris-HCl–100 mM NaCl, pH 7.5) for 30 min under gentle stirring. The QAE-Sephadex was filtered on a funnel overlaid with nylon cloth, washed with 10 L of the equilibration buffer, resuspended in a buffer containing 10 mM Tris-HCl, 100 mM NaCl, and 20% (v/v) glycerol, pH 7.5, and packed into a column (10 × 30 cm). Factor V was eluted with 10 mM Tris-HCl, 500 mM NaCl, and 10 mM benzamidine, pH 7.5. The eluate was dialyzed against 10 mM Tris-HCl, 100 mM NaCl, 10 mM benzamidine, and 20% (v/v) glycerol, pH 7.5, and applied to a DEAE-Sephadex column (5 × 30 cm) equilibrated with the dialysis buffer. The column was developed at 4 °C with a linear gradient from 100 mM NaCl in 20 mM Tris-HCl, 10 mM benzamidine, and 20% (v/v) glycerol, pH 7.5, to 300 mM NaCl in 20 mM Tris-HCl–100 mM benzamidine, pH 7.5 (3.0 L/reservoir). Factor V activity eluted at 200–250 mM NaCl. The factor V containing fractions were diluted with an equal volume (normally 1 L) of a buffer containing 20 mM potassium phosphate, 20 mM Tris-HCl, 150 mM sodium oxalate, and 10 mM benzamidine, pH 7.0. Calcium chloride (2 M) was added slowly with stirring to a final concentration of 100 mM. This mixture was stirred for 30 min at 4 °C. The calcium oxalate and calcium phosphate precipitate was collected by centrifugation for 20 min at 2000*g*. Factor V activity was eluted with 2 times 250 mL of 200 mM potassium phosphate, 10 mM benzamidine, and 2 mM DFP, pH 7.0. The eluate was saturated to 30% with ammonium sulfate, stirred for 30 min at 4 °C, and then centrifuged for 20 min at 2000*g*. Factor V precipitated when the supernatant was saturated to 60% with ammonium sulfate. The precipitate was spun down and resuspended in a minimal volume of 20 mM Tris-HCl, 10 mM benzamidine, and 1 mM DFP, pH 7.5, and applied to a column (5 × 100 cm) of Ultrogel AcA 22 equilibrated in 50 mM Tris-HCl, 400 mM NaCl, and 10 mM benzamidine, pH 7.5. Factor V appeared after about 700 mL of eluate. The fractions of highest specific activity were pooled and stored at −80 °C.

Bovine factor Va (unfractionated factor Va) was obtained by incubating 0.3 mg/mL factor V with thrombin (0.2 μg/mL) in 50 mM Tris-HCl, 100 mM NaCl, and 2 mM $CaCl_2$, pH 7.5, at 37 °C for 30 min. The activation of factor V was followed by assay of factor Va activity and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After complete activation, the factor Va solution was diluted in 50 mM Tris-HCl, 100 mM NaCl, 2 mM $CaCl_2$, and 0.5 mg/mL ovalbumin, pH 7.5, 1:50, and stored at −80 °C. Factor Va was stable for at least 6 months.

The factor Va subunits with molecular weights of 80 000 (factor Va LC) and 94 000 (factor Va HC) were isolated by a modification of the method of Esmon (1979). Factor Va (30 mg) in 20 mM Tris-HCl, 50 mM NaCl, and 2 mM EDTA, pH 7.5, was applied to a SP-Sephadex column (1.5 × 30 cm) coupled to a QAE-Sephadex column (1.5 × 30 cm), both equilibrated with 20 mM Tris, 50 mM NaCl, and 2 mM EDTA, pH 7.5. Subsequently, the SP-Sephadex and QAE-Sephadex columns were developed separately with linear gradients from 50 mM NaCl to 200 mM NaCl and from 50 mM NaCl to 500 mM NaCl (150 mL/reservoir), respectively. The factor Va LC was eluted from the SP-Sephadex column (100 mM NaCl), and the factor Va HC was eluted from the QAE-Sephadex column (350 mM NaCl).

Phospholipid Vesicle Preparations. Phospholipid vesicles were made from a mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphoserine and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine in a buffer containing 50 mM Tris-HCl–100 mM NaCl, pH 7.5, as described earlier (Rosing et al., 1980).

Reconstitution of Factor Va from Its Subunits (Factor Va LC and Factor Va HC). Factor Va LC (0.3 mg/mL) in 50 mM Tris-HCl, 100 mM NaCl, 20 mM $CaCl_2$, and 0.5 mg/mL ovalbumin, pH 7.5, and factor Va HC (0.4 mg/mL) in the same buffer were incubated at 37 °C. The time course of factor Va activity was followed by a factor V assay.

Factor V Assay. Factor V was assayed in a one-stage assay utilizing purified bovine coagulation factors. A sample (50 μL) was incubated with 50 μL of factor Xa (0.1 nM), 50 μL of a mixture containing phospholipid (100 μM) and human fibrinogen (4 mg/mL), and 50 μL of 25 mM $CaCl_2$ for 30 s

[2] Abbreviations: S 2238, D-Phe-L-Pip-L-Arg-*p*-nitroanilide; S 2222, *N*-benzoyl-L-Ile-L-Glu-[Y-Or]-Gly-L-Arg-*p*-nitroanilide; DFP, diisopropyl fluorophosphate; RVV-X, purified factor X activator from Russel's viper venom; LC, light chain; HC, heavy chain; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; QAE, [diethyl(2-hydroxypropyl)amino]ethyl; DEAE, diethylaminoethyl; SP, sulfopropyl.

at 37 °C. The assay was initiated by the addition of 50 μL of prothrombin (5 μM). The time required for clot formation was measured. Samples were diluted in 50 mM Tris-HCl, 100 mM NaCl, and 2 mM $CaCl_2$, pH 7.5, containing ovalbumin (0.5 mg/mL). The purified coagulation factors were in the same buffer. Pooled bovine oxalated plasma was used for the construction of standard curves. The assay had a functional range from 0.05 to 10 milliunits/mL (clotting times from 300 to 60 s). One unit of factor V was defined as the amount present in 1 mL of bovine plasma.

Protein Concentrations. Factor Xa concentrations were determined by active site titration with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride according to Smith (1973). The molar concentrations of the other proteins were calculated from the following molecular weights and $E^{1\%,1cm}_{280nm}$: prothrombin 72000, 15.5 (Owen et al., 1974); prethrombin 1 50200, 19.2 (Owen et al., 1974); factor V 330000, 9.6 (Nesheim et al., 1979a); factor Va LC 80000, 15.8; factor Va HC 94000, 9.6 (Esmon, 1979).

Measurement of Rates of Prothrombin Activation. To determine the K_d and the stoichiometry of complex formation between factor Xa and factor Va, we used a method in which initial rates of prothrombin activation are a measure for the amount of factor Xa–Va complex present in a reaction mixture (for a description of this method, see below). Rates of prothrombin activation were calculated from the amounts of thrombin determined after different time intervals with the synthetic chromogenic substrate S 2238 as described previously (Rosing et al., 1980). The conversion of S 2238 by thrombin was followed by measuring the absorbance change on an Aminco DW-2 spectrophotometer operating in the dual-wavelength mode ($\lambda_{mono\,1}$ = 405 nm and $\lambda_{mono\,2}$ = 500 nm).

Method To Determine Binding Interaction between Factor Xa and Factor Va from Kinetic Measurements. Kinetic studies on prothrombin activation (Nesheim et al., 1979b) show that in the presence of factor Va, prothrombin activation is catalyzed by an equimolar complex between factor Xa and factor Va. The initial assumption in this study is that the formation of the complex is described by

$$\text{factor Xa} + \text{factor Va} \rightleftharpoons \text{factor Xa–Va} \quad (1)$$

The dissociation constant of the factor Xa–Va complex is

$$K_d = [\text{factor Va}]_{free}[\text{factor Xa}]_{free}/[\text{factor Xa–Va}] \quad (2)$$

When a fixed amount of factor Xa is titrated with factor Va

$$[\text{factor Xa}]_{free} = [\text{factor Xa}]_0 - [\text{factor Xa–Va}] \quad (3)$$

in which $[\text{factor Xa}]_0$ is the total concentration of factor Xa present. Substituting eq 3 into eq 2 gives

$$K_d = [\text{factor Va}]_{free}([\text{factor Xa}]_0 - [\text{factor Xa–Va}])/[\text{factor Xa–Va}] \quad (4)$$

which can be rearranged into

$$1/[\text{factor Xa–Va}] = (1/[\text{factor Va}]_{free})(K_d/[\text{factor Xa}]_0) + 1/[\text{factor Xa}]_0 \quad (5)$$

When prothrombin is added to a reaction mixture in which factor Xa, factor Va, and the factor Xa–Va complex are in equilibrium, conditions can be chosen such that the rate of prothrombin activation is proportional to the amount of factor Xa–Va complex present, since noncomplexed factor Xa catalyzes prothrombin activation at a rate negligible to that of the factor Xa–Va complex (Rosing et al., 1980). In that case, the rate of prothrombin activation is

$$v = c[\text{factor Xa–Va}] \quad (6)$$

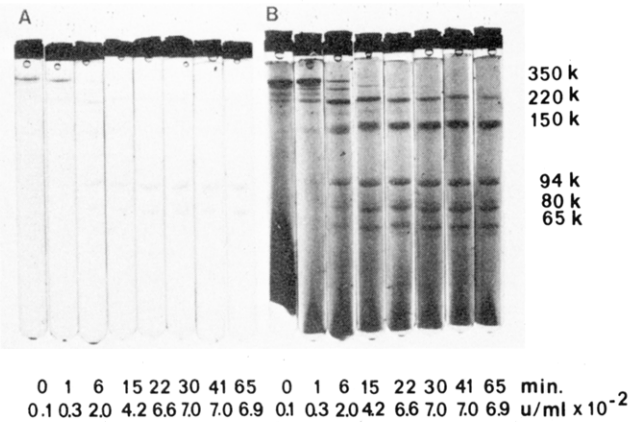

FIGURE 1: Activation time course of factor V by thrombin. Factor V (0.5 mg/mL) was incubated with thrombin (0.1 μg/mL) at 37 °C in 0.05 M Tris-HCl–0.1 M NaCl, pH 7.5. Samples were removed from the reaction mixture at the times given below the gels and assayed for factor V activity. At the same time intervals aliquots (50 μL) of the incubation mixture were added to 50 μL of 2% sodium dodecyl sulfate, 10% (v/v) mercaptoethanol, and 50 mM EDTA, pH 7.8, and kept for 2 min at 100 °C. Samples were electrophoresed on 7.5% $NaDodSO_4$–acrylamide gels and stained with Coomassie blue (A) and with periodic acid–Schiff's reagent (B).

in which *c* is a constant determined by the concentration of prothrombin present and the kinetic parameters of prothrombin activation by the factor Xa–Va complex. When all factor Xa present is complexed with factor Va, [factor Xa–Va] = $[\text{factor Xa}]_0$, and the maximal rate of prothrombin activation is equal to

$$V_{max} = c[\text{factor Xa}]_0 \quad (7)$$

Substituting eq 6 and 7 into eq 5 gives

$$1/v = (1/[\text{factor Va}]_{free})(K_d/V_{max}) + 1/V_{max} \quad (8)$$

When $[\text{factor Xa}]_0 \ll K_d$, the amount of factor Va required for saturating factor Xa is in large excess over $[\text{factor Xa}]_0$, and $[\text{factor Va}]_{free} \simeq [\text{factor Va}]_0$, where $[\text{factor Va}]_0$ is the total concentration of factor Va present. So

$$1/v = (1/[\text{factor Va}]_0)(K_d/V_{max}) + 1/V_{max} \quad (9)$$

An equation similar to 9 can be derived when a fixed amount of factor Va is titrated with factor Xa

$$1/v = (1/[\text{factor Xa}]_0)(K_d/V_{max}) + 1/V_{max} \quad (10)$$

A plot of $1/v$ vs. either $1/[\text{factor Va}]_0$ or $1/[\text{factor Xa}]_0$ is linear with an intercept at the ordinate equal to $1/V_{max}$ and at the abscissa equal to $-1/K_d$. K_d and V_{max} were determined by statistical analysis of the data as described by Eisenthal & Cornish-Bowden (1974).

Results

Comments on Purification of Factor V and Isolation of End Products of Thrombin-Catalyzed Activation of Factor V. Factor V prepared by a modification of the method of Esmon (1979) as described under Materials and Methods is a single-chain polypeptide with an apparent M_r of 350000 as determined with $NaDodSO_4$ electrophoresis (Figure 1). In contrast to Esmon (1979) we did not observe a tightly spaced doublet. The absence of the doublet in our preparations is due to the different way in which we obtained the blood as starting material (i.e., venipunction of cows treated with phenprocoumen vs. slaughterhouse blood). We have purified factor V approximately 2000-fold [sp act. 24 units mL^{-1} (absorbance unit at 280 nm)$^{-1}$] with a 20% yield.

The factor V preparation appears to be free of phospholipid contamination. Phospholipid as estimated by organic phos-

phate measurement according to the method of Böttcher et al. (1961) was undetectable in a sample containing 2 mg of factor V (less than 0.5 mol of phospholipid/mol of factor V). In addition, incubation of factor Va (1 μmol/L) with phospholipase A_2 from *Naja naja* (2 IU/mL) in the presence of 10 mM $CaCl_2$ for 30 min at 37 °C did not result in a change in factor Va activity as measured in the absence of phospholipid under the conditions as described in the legend to Figure 2A. When incubated with thrombin, our factor V preparations are activated 75–100-fold. The lower increase in specific activity reported by Esmon (1979), 10–16-fold, can be accounted for by the different factor V assays used. Analysis of the fully activated factor V by $NaDodSO_4$ gel electrophoresis and staining of the gels with periodic acid–Schiff's reagent (PAS) and Coomassie blue reveal that activation of factor V by thrombin gives rise to four polypeptides with M_r 150 000, 94 000, 80 000, and 65 000 (Figure 1). The polypeptides with M_r 150 000 and 65 000 could only be detected by PAS staining. The four polypeptides were isolated by ion-exchange chromatography as described under Materials and Methods. The polypeptide with M_r 80 000 eluted from the SP-Sephadex column (0.10 M NaCl). The polypeptides with M_r 65 000, 94 000, and 150 000 eluted from the QAE-Sephadex at 0.15, 0.35, and 0.40 M NaCl, respectively. No factor Va activity could be detected in any of the column fractions. As reported by Esmon (1979), factor Va activity could be restored by mixing the SP-Sephadex fraction that contains the polypeptide with M_r 80 000 (factor Va LC) with the QAE-Sephadex fraction containing the polypeptide with M_r 94 000 (factor Va HC) in the presence of 20 mM $CaCl_2$. Therefore, we consider the polypeptides with M_r 65 000 and 150 000 as activation peptides.

Binding Interaction between Factor Xa and Factor Va in Solution Inferred from Kinetics of Prothrombin Activation. The dissociation constant (K_d) of the factor Xa–Va complex was determined from kinetics of prothrombin activation as described under Materials and Methods. Initial rates of prothrombin activation were measured when a fixed amount of factor Xa is titrated with factor Va and vice versa. True initial rates were measured since linearity of prothrombin activation was obtained at all factor Xa and factor Va concentrations used and the maximal amount of prothrombin converted never exceeded 2% of the prothrombin added. Factor Xa and factor Va were incubated for 10 min at 37 °C in the presence of Ca^{2+} ions prior to addition of prothrombin, since at the lowest factor Xa and factor Va concentrations employed in our experiments, complex formation was reached in 5 min.

The results of the titration experiments are shown in Figure 2A as a plot of the reciprocal value of the initial rate of prothrombin activation vs. the reciprocal concentration of the varied component (factor Va or factor Xa).[3] The respective dissociation constants and maximal rates of thrombin formation are $K_d = 3.5 \times 10^{-9}$ M and $V_{max} = 5.4$ min^{-1} for a fixed amount of factor Xa and $K_d = 3.1 \times 10^{-9}$ M and $V_{max} = 5.1$ min^{-1} for a fixed amount of factor Va. Since in both titrations the amount of varied component was always in large excess over the amount of fixed component, a negligible fraction of the ligand is present in the complex, which justifies calculation of the dissociation constants from the concentration of added ligand. The same K_d and V_{max} values obtained in titrations of factor Xa with factor Va and titrations of factor Va with factor Xa show that in solution an equimolar complex is formed between factor Xa and factor Va.

[3] When a fixed amount of factor Va was titrated with factor Xa, a correction was made for the contribution of noncomplexed factor Xa to the rate of thrombin formation. At the factor Xa concentration required to saturate 50% of the factor Va present, the contribution of noncomplexed factor Xa to the rate of prothrombin activation was 6% of the total activity measured.

Table I: Influence of Prothrombin and Prethrombin 1 on the K_d of the Factor Xa–Va Complex

substrate	concn (μM)	K_d[a] (nM)
prothrombin	0.09	2.5
	0.20	3.7
	0.40	3.5
	1.40	4.9
	2.00	3.8
	3.00	4.6
prethrombin 1	0.20	5.0
	1.00	4.6

[a] The dissociation constants were calculated from double-reciprocal plots obtained by titrating factor Xa with factor Va as described in the legend to Figure 2A.

Effect of Ca^{2+} on Interaction between Factor Xa and Factor Va in Solution. As shown by Rosing et al. (1980), the rate of prothrombin activation by factor Xa in the presence of factor Va increases to an optimum when the Ca^{2+} concentration is increased from 0 to 3 mM. At higher Ca^{2+} concentrations the rate of prothrombin activation decreases again. The data presented in Figure 3 confirm these findings. In this figure the maximal rate of prothrombin activation is plotted as a function of the Ca^{2+} concentration. Maximal rates of prothrombin activation were calculated from double-reciprocal plots obtained by titrating factor Xa with factor Va in the presence of varying amounts of Ca^{2+} ions. The same Ca^{2+} dependence was found when prethrombin 1 or decarboxyprothrombin was used instead of prothrombin (data not shown). Figure 3 also shows that the dissociation constant of the factor Xa–Va complex is independent of the Ca^{2+} concentration between 0.5 and 30 mM. Since at Ca^{2+} concentrations below 0.5 mM rates of prothrombin activation were too low to determine a K_d, it could not be established whether there is an absolute Ca^{2+} requirement for the interaction of factor Xa with factor Va.

Effect of Prothrombin on Interaction between Factor Xa and Factor Va in Solution. Prothrombin interacts with factor Va, presumably through the fragment 2 region of the prothrombin molecule (Esmon et al., 1974). To examine whether an interaction between prothrombin and factor Va affects the formation of the factor Xa–Va complex in solution, we performed titrations of factor Xa with factor Va at various prothrombin concentrations. Factor Xa (0.32 nM) was titrated with factor Va at prothrombin concentrations between 0.09 and 3.0 μM. The prothrombin concentrations are (far) below the K_m for prothrombin (Rosing et al., 1980). The fact that the double-reciprocal plots of rates of prothrombin activation vs. total factor Va added are linear at all prothrombin concentrations used indicates that initial rates of prothrombin activation are also under these conditions a measure for the amount of factor Xa–Va complex present.

The K_d values for the factor Xa–Va complex calculated from these plots were independent of the amount of prothrombin present (Table I). From these results we conclude that the binding interaction between factor Xa and factor Va as inferred from kinetics is not affected by an interaction of prothrombin with factor Va and/or factor Xa.

Effect of 40% DOPS/60% DOPC Phospholipid Vesicles on Interaction between Factor Xa and Factor Va. The binding

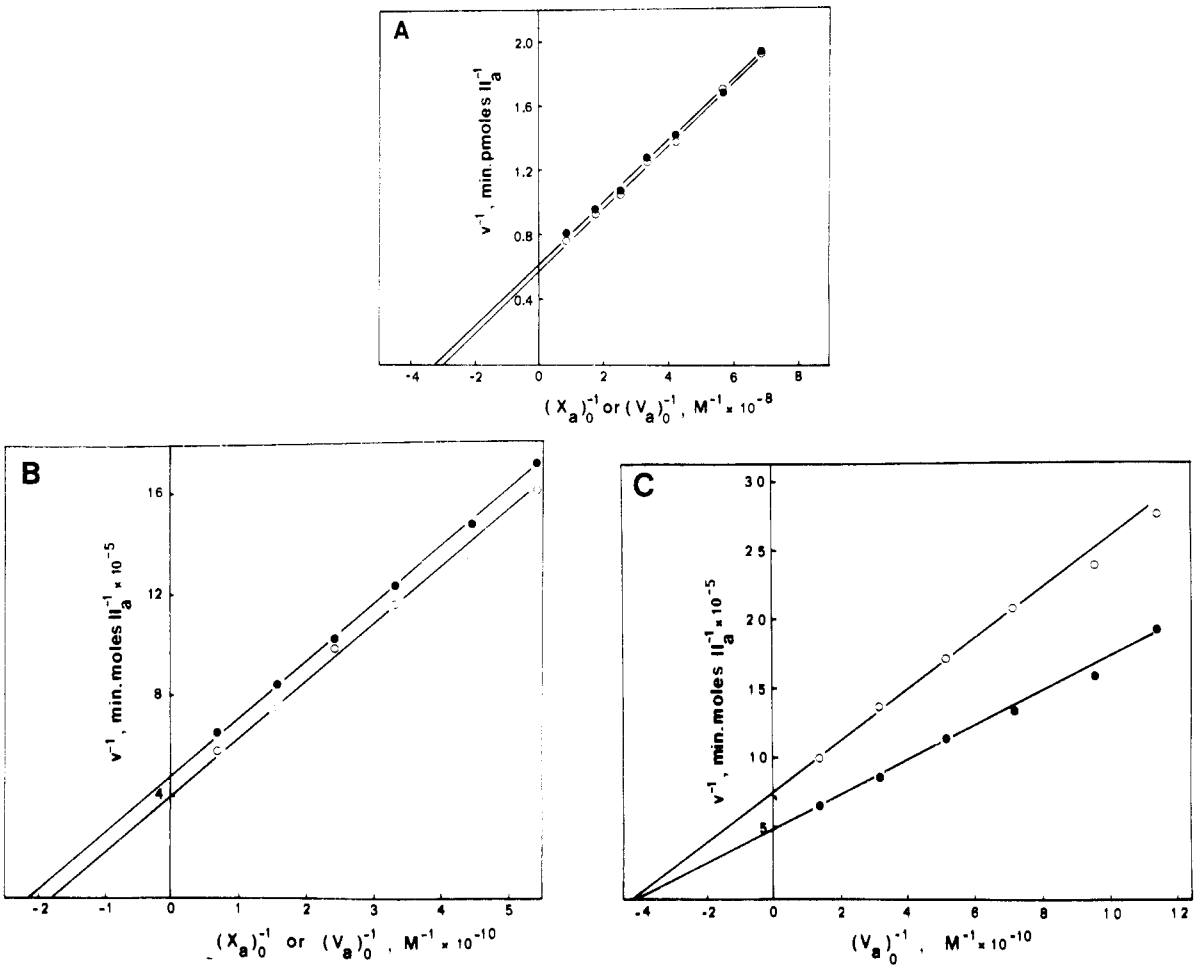

FIGURE 2: Double-reciprocal plots of binding interaction of factor Va with factor Xa. Factor Xa and factor Va were incubated for 10 min at 37 °C either in the absence of phospholipid and 3 mM $CaCl_2$ or in the presence of phospholipid and 10 mM $CaCl_2$ in 1.0 mL of a buffer containing 50 mM Tris-HCl, 100 mM NaCl, and ovalbumin (0.5 mg/mL), pH 7.5. The prothrombin activation was started by the addition of 10 μL of prothrombin to give final concentrations as given below. After 2 and 4 min, samples were taken and assayed for thrombin as described under Materials and Methods. Each point in the double-reciprocal plot is the average of two independent measurements. Panel A gives the data for mixtures consisting of either 3.5×10^{-10} M factor Xa and $(1.2–12.0) \times 10^{-9}$ M factor Va (○) or 3.5×10^{-10} M factor Va and $(1.2–12.0) \times 10^{-9}$ M factor Xa (●). Final prothrombin concentration is 0.2 μM. Panel B gives the data for mixtures consisting of either 2.1×10^{-12} M factor Xa, $(0.15–1.5) \times 10^{-10}$ M factor Va, 20 μM phospholipid (40% DOPS/60% DOPC), and 10 mM $CaCl_2$ (○) or 2.0×10^{-12} M factor Va, $(0.15–1.5) \times 10^{-10}$ M factor Xa, 20 μM phospholipid (40% DOPS/60% DOPC), and 10 mM $CaCl_2$ (●). Final concentration of prothrombin is 1.0 μM. Panel C gives the data for mixtures consisting of 2.1×10^{-12} M factor Xa, $(0.7–7.0) \times 10^{-11}$ M factor Va, 5 μM phospholipid (40% DOPS/60% DOPC), 10 mM $CaCl_2$, and prothrombin concentrations of 0.05 μM (○) and 1.0 μM (●).

data obtained in the absence of phospholipid show that factor Xa and factor Va form an equimolar complex with a dissociation constant of 3.3×10^{-9} M. Since both factor Xa and factor Va bind with a high affinity to negatively charged phospholipid vesicles (Nelsestuen & Broderius, 1977; Bloom et al., 1979), it is likely that the presence of phospholipid affects in some manner the dissociation constant of the factor Xa–Va complex. From the fact that much lower amounts of factor Va were required to optimize prothrombin activation in the presence of phospholipid than in solution, Rosing et al. (1980) suggested that phospholipid promotes the formation of the factor Xa–Va complex. Also, the kinetic studies of Nesheim et al. (1979b, 1981) and the binding studies of Tracy et al. (1981) indicate that factor Xa has an increased affinity toward phospholipid-bound factor Va.

To appreciate the assembling function of phospholipid in the formation of the factor Xa–Va complex, we determined apparent dissociation constants at different phospholipid concentrations by the kinetic method as described under Materials and Methods. Figure 2B shows the result of a typical experiment carried out in the presence of 20 μM phospholipid (40% DOPS/60% DOPC). Titrations were carried out with both factor Xa and factor Va as the limiting component. The respective apparent dissociation constants and maximal rates of prothrombin activation are $K_d = 5.5 \times 10^{-11}$ M and $V_{max} = 1230$ min^{-1} for a fixed amount of factor Xa and $K_d = 4.6 \times 10^{-11}$ M and $V_{max} = 1050$ min^{-1} for a fixed amount of factor Va.[4] Also in these experiments, the amount of ligand added was always in large excess over the amount of limiting component. Therefore, it was justified to express the apparent K_d in terms of total added ligand. Under the same conditions but in the absence of phospholipid, the rate of thrombin formation is negligible as compared to the rate of thrombin formation in the presence of phospholipid. Therefore, the prothrombin activation measured is restricted to reactions taking place at the phospholipid surface and the result of interactions between factor Xa and factor Va at that

[4] When a fixed amount of factor Va was titrated with factor Xa, a correction was made for the contribution of noncomplexed factor Xa to the rate of thrombin formation. At the factor Xa concentration required to saturate 50% of the factor Va present, the contribution of noncomplexed factor Xa to the rate of prothrombin activation was 8% of the total activity measured.

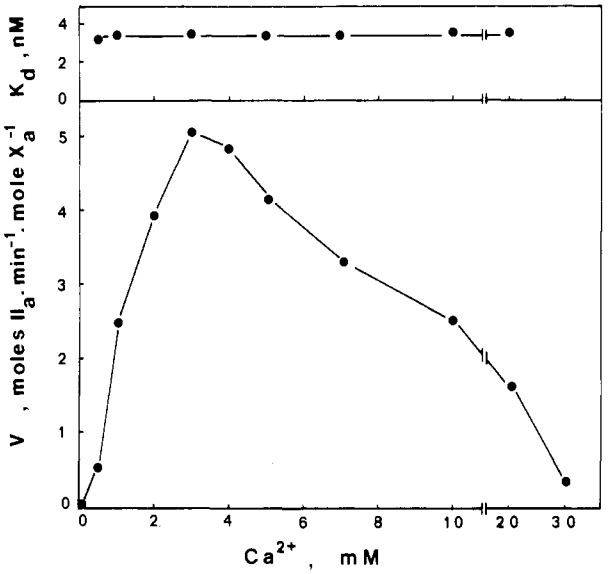

FIGURE 3: Effect of Ca^{2+} concentration on the V_{max} of thrombin formation and the dissociation constant of the factor Xa–Va complex. Factor Xa (3.5×10^{-10} M) was titrated with factor Va in the presence of varying amounts of Ca^{2+} as described in the legend to Figure 2A. The ionic strength of the reaction mixtures was kept constant by varying the NaCl concentration. The maximal rates of prothrombin activation (V, vertical axis, lower panel) and the dissociation constant (K_d, vertical axis, upper panel) were determined from double-reciprocal plots of rate of thrombin formation vs. added factor Va.

Table II: Effect of Phospholipid Vesicles (40% DOPS/60% DOPC) on the Apparent Dissociation Constant of the Factor Xa–Va Complex

phospholipid concn (μM)	prothrombin concn (μM)	K_d(app)[a] (M)	V_{max}[b]
2	1.0	1.39×10^{-11}	1190
5	1.0	2.30×10^{-11}	1000
10	1.0	2.84×10^{-11}	1280
25	1.0	4.51×10^{-11}	1600
50	1.0	9.29×10^{-11}	1380
100	1.0	15.80×10^{-11}	1100
125	2.0	18.90×10^{-11}	1590
150	3.0	27.00×10^{-11}	1580
300	6.0	52.20×10^{-11}	1780

[a] The apparent dissociation constants and V_{max} were calculated from double-reciprocal plots obtained by titrating factor Xa with factor Va as described in the legend to Figure 2B. [b] The rates are expressed in moles of factor IIa per minute per mole of factor Xa.

surface. The data obtained in Figure 2B show that a phospholipid surface promotes the formation of the factor Xa–Va complex by decreasing the dissociation constant about 100-fold.

Binding parameters obtained from double-reciprocal plots of titrations of a fixed amount of factor Xa with factor Va in the presence of varying amounts of phospholipid are listed in Table II. The apparent dissociation constant increases when the phospholipid concentration is raised. At phospholipid concentrations higher than 25 μM the increase of the dissociation constant is linear with the amount of phospholipid present.

Since the K_m for prothrombin varies with the phospholipid concentration (Rosing et al., 1980), the experiments presented in Table II were performed at variable amounts of prothrombin. The prothrombin concentrations employed were at least 4 times higher than the apparent K_m for prothrombin in order to approach the true V_{max} of prothrombin activation as close as possible. As shown in Table II, the V_{max} values

Table III: Dissociation Constant Determined by Kinetics for Interaction of Factor Xa with Factor Va in the Presence of Phospholipid Vesicles[a] of Varying Phosphatidylserine (PS) Content

fraction PS (%)	total lipid concn (μM)	K_d[b] (M)	prothrombin concn (μM)	V_{max}[b]
5	10	33×10^{-11}	0.2	1040
	20	25×10^{-11}	0.5	950
	50	28×10^{-11}	1.0	1200
	100	28×10^{-11}	2.0	1200
	150	28×10^{-11}	2.0	1200
10	10	8.0×10^{-11}	0.2	1300
15	10	3.8×10^{-11}	0.2	1440
20	10	2.9×10^{-11}	0.2	1490
30	10	3.0×10^{-11}	0.2	1400
40	10	3.0×10^{-11}	0.2	1350

[a] Phospholipid vesicles were made as described under Materials and Methods. [b] The K_d and V_{max} were calculated from double-reciprocal plots obtained by titrating factor Xa (2.0×10^{-12} M) with factor Va as described in the legend to Figure 2B. The rates are expressed in moles of factor IIa per minute per mole of factor Xa.

at different phospholipid concentrations are about equal. From this and the assumption that all factor Xa is saturated with substrate, we conclude that all factor Xa present in the reaction mixtures participates in a membrane-bound factor Xa–Va complex.

It can be argued that prothrombin might interfere with the formation of the factor Xa–Va complex at the phospholipid surface either by interaction with factor Xa and/or factor Va or by competition with factor Xa and/or factor Va for binding sites at the phospholipid surface. Since a 20-fold variation of the prothrombin concentration did not affect the apparent dissociation constant (Figure 2C), we conclude that prothrombin does not interfere with the assembly of the factor Xa–Va complex at the phospholipid surface. The increase of the V_{max} from 670 to 1000 mol of thrombin min^{-1} (mol of factor Xa)$^{-1}$ presumably reflects substrate saturation of prothrombin activation, since the apparent K_m for prothrombin in the presence of 5 μM phospholipid is 0.06 μM. A phospholipid concentration of 5 μM was chosen for this experiment since it provides a limited number of binding sites for factor Xa and factor Va, which would have made competition, if occurring, more easily detectable.

Interaction between Factor Xa and Factor Va at Phospholipid Vesicles with Varying Phosphatidylserine Content. It has been demonstrated that binding of both factor Xa and factor Va to membranes requires the presence of negatively charged phospholipids (Bloom et al., 1979; Nelsestuen & Broderius, 1977). Nelsestuen & Broderius (1977) found that below 20% phosphatidylserine the factor Xa binding capacity was directly proportional to the phosphatidylserine content. The data of Bloom et al. (1979) suggest that below 25% phosphatidylserine the binding capacity of factor Va is also directly proportional to the phosphatidylserine content. The characteristics of the formation of the factor Xa–Va complex at membranes containing varying amounts of phosphatidylserine are shown in Table III. The results show that the K_d(app) of the factor Xa–Va complex decreases up to about 20% phosphatidylserine and is then constant to 50% phosphatidylserine. The maximal rate of prothrombin activation did not change with the phosphatidylserine content.

Table III also shows that at high phosphatidylserine content (40%) the K_d of the factor Xa–Va complex increases proportional to the phospholipid concentration. At low phosphatidylserine content (5%) the K_d(app) is independent of the

Table IV: Effect of Factor V Activation Fragments on the Binding Interaction between Factor Va and Factor Xa[a]

	added component					
	factor Xa (nM)	factor Va (nM)	fragments	concn (nM)	K_d (nM)	V_{max}[b]
1	0.35	1.0–10.0	none		4.0	4.7
2	0.35	none	restored factor Va	1.0–10.0	3.5	4.3
3	0.35	1.0–10.0	factor Va LC	50	3.7	5.0
4	0.35	1.0–10.0	factor Va HC	100	3.3	4.5

[a] The K_d and V_{max} values were determined from double-reciprocal plots of rates of thrombin formation vs. added factor Va or restored factor Va. Further experimental details are described in the legend to Figure 2A. [b] The rates are expressed in moles of factor IIa per minute per mole of factor Xa.

phospholipid concentrations, ranging from 50 to 150 μM. It is apparent that almost all factor Xa added binds to the phospholipid surface and can be saturated with factor Va, since maximal rates of thrombin formation were found to be independent of the phospholipid concentration and slightly less than those observed for vesicles with a high phosphatidylserine content.

Studies on Interaction of Factor Va Subunits with Factor Xa. In our studies we have used an unfractionated factor Va preparation that contains the biologically active factor Va, composed of two polypeptide chains with M_r 80 000 (factor Va LC) and 94 000 (factor Va HC) and activation peptides with M_r 150 000 and 65 000. It is of interest to investigate whether the presence of those activation peptides affects the interaction between factor Va and factor Xa and to examine whether restoration of biological activity correlates with the Ca^{2+}-mediated complex formation between factor Va LC and factor Va HC, showing the same binding characteristics toward factor Xa as does the "native" factor Va. Titration of factor Xa (0.5×10^{-9} M) with restored factor Va [$(1.0–10) \times 10^{-9}$ M] was performed as described in the legend to Figure 2A. The K_d and V_{max} values calculated from a double-reciprocal plot of the rate of prothrombin activation vs. added factor Va HC–factor Va LC complex are presented in Table IV. A comparison between entries 1 and 2 indicates that (a) the activation peptides have no effect on the interaction between factor Xa and factor Va and (b) the binding characteristics of restored factor Va are the same as those of native factor Va. These findings strongly support the observations made by Esmon (1979) that two nonidentical peptides derived from the procofactor are required for biological function. These observations also suggest that the two-chain structure of factor Va is required for binding factor Xa and/or prothrombin.

Whether the interaction between factor Xa and one of the peptides is impaired because of the absence of the other peptide can easily be demonstrated. If one of the peptides forms a complex with factor Xa, this peptide is likely to be inhibitory, since it will competitively removes factor Xa from interaction with factor Va, thereby reducing the concentration of the functional enzyme (factor Xa–Va complex) in prothrombin activation. Table IV, entries 3 and 4, clearly demonstrates that in the presence of a large molar excess of factor Va LC or factor Va HC over factor Va, the dissociation constants and V_{max} values are similar to those found in the absence of each of the chains. It is apparent that neither factor Va HC nor factor Va LC is able to compete with factor Va in a complex formation with factor Xa. From these data we conclude that factor Xa binding to factor Va requires a Ca^{2+}-mediated interaction between the polypeptides with M_r 80 000 and 94 000 of which factor Va is composed.

Discussion

The interaction between factor Xa and factor Va in the presence of phosphatidylserine–phosphatidylcholine vesicles and platelets has been studied in several laboratories (Nesheim et al., 1979b; Kane et al., 1980; Tracy et al., 1981). The results obtained suggest a coordinate binding of factor Xa and factor Va to phospholipid and platelets and the formation of a stoichiometric (1:1) complex. The dissociation constant for factor Xa dissociation from the phospholipid-bound complex ($K_d \sim 10^{-10}$ M) is several orders of magnitude less than the dissociation constant for factor Xa–phospholipid interaction ($K_d \sim 10^{-6}$ M) (Nelsestuen & Broderius, 1977; Bloom et al., 1979). On the basis of these observations, it has been postulated that binding of factor Va to phospholipid increases the binding affinity for factor Xa. Therefore, relatively high concentrations of factor Xa are obtained in the vicinity of phospholipid vesicles and platelets when factor Va is present (Nesheim et al., 1981). There exists, however, no insight in the interactions responsible for the increased affinity of factor Xa for phospholipid-bound factor Va. The question is to what extent factor Xa–phospholipid, factor Va–phospholipid, and protein–protein interactions contribute to the increased affinity of phospholipid-bound factor Va for factor Xa.

The studies presented in this paper were initiated in order to disclose the contribution of both protein–protein and protein–phospholipid interactions to the high affinity of the phospholipid-bound factor Va for factor Xa. The method used to study the interaction between factor Xa and factor Va is based on the assumption that a factor Xa–Va complex is the functional enzyme in prothrombin activation (Nesheim et al., 1979b; Rosing et al., 1980) and that the rates of prothrombin activation by factor Xa in the presence of factor Va are proportional to the amount of factor Xa–Va complex in the reaction mixture. In the absence of phospholipid, titrations of a fixed amount of factor Xa with factor Va or vice versa yield at saturation the same maximal rates for prothrombin activation per mole of fixed component (Figure 2A). The same observation was made in titration experiments in the presence of phospholipid (Figure 2B). Since factor Va is a nonenzymatic component, these results indicate that in both the absence and presence of phospholipid, the catalytic unit in the prothrombin activation is a stoichiometric (1:1) complex of factor Xa and factor Va.

Because prothrombin has the ability to interact with factor Va through the fragment 2 region of the prothrombin molecule (Esmon et al., 1974) and factor Va amplifies the turnover of prothrombin by factor Xa by not yet disclosed means (Rosing et al., 1980), the question arises whether prothrombin interferes with the interaction between factor Xa and factor Va. Given the following observations, it seems unlikely that prothrombin participates in the formation of the factor Xa–Va complex: (1) The dissociation constant of the factor Xa–Va complex is independent of the prothrombin concentration when the molar ratios of prothrombin to factor Va were varied from 9 to 3000 (Table I), (2) in the presence of phospholipid, the apparent dissociation constant was also independent on the prothrombin concentration (Figure 2C), and (3) Nesheim et al. (1981) showed that the binding parameters of the interaction between factor Xa and phospholipid-bound factor Va inferred from alterations in the rate of hydrolysis of S 2222 are similar to those obtained with prothrombin as a substrate.

Rosing et al. (1980) demonstrated that the generation of thrombin, upon incubation of prothrombin with factor Xa and

Table V: Apparent Dissociation Constant for Interaction of Factor Xa with Factor Va and Corresponding Factor Va Concentration on the Phospholipid Surface and Factor Va Concentration in Solution at Varying Phospholipid Concentrations and Phosphatidylserine Content of DOPC/DOPS Vesicles

phospholipid	concn (μM)	K_d(app) $\times 10^{11}$ (M)	[factor Va]$_{free}$ $\times 10^{11}$ (M)	factor Va concn on phospholipid surface (μmol/mol)
40% DOPS/	2	1.4	0.99	2.0
60% DOPC	5	2.3	1.15	2.3
	10	2.9	0.95	1.9
	25	4.5	0.75	1.5
	50	9.3	0.85	1.7
	100	15.8	0.75	1.5
	125	18.9	0.73	1.5
	150	27.0	0.87	1.7
	300	52.2	0.86	1.7
5% DOPS/	10	33.0	31.7	1.3
95% DOPC	20	25.0	18.5	0.8
	50	28.0	23.3	1.0
	100	28.0	20.0	0.8
	150	28.0	17.5	0.7

factor Va, strongly depends on the Ca^{2+} concentration, with an optimal Ca^{2+} concentration around 3 mM. Our results suggest that Ca^{2+} ions are not participating in the factor Xa–Va complex formation but presumably affect the enzymatic properties of the complex, because the K_d of the complex is independent of the Ca^{2+} concentration. The same Ca^{2+} dependence was found when substrates lacking γ-carboxyglutamic acid residues were used. This suggests a role of Ca^{2+} in the enzymatic properties of the complex apart from its interaction with the γ-carboxyglutamic acid residues of the substrate.

Phospholipid does decrease the dissociation constant of the factor Xa–Va complex. When a fixed amount of factor Xa is titrated with factor Va in the presence of 25 μM phospholipid (40% DOPS/60% DOPC) and 10 mM $CaCl_2$, the K_d is 5.7×10^{-11} M, which is about 2 orders of magnitude less than the K_d determined in the absence of phospholipid. A considerable increase of the K_d is measured when the amount of phospholipid is increased from 25 to 300 μM (Table II). The K_d increases almost linearly with the phospholipid concentration.

We demonstrated that the K_d is also a function of the phosphatidylserine content of the membrane. The dependence of K_d on the phosphatidylserine content may reflect the phospholipid-binding affinities of factor Xa and/or factor Va, because binding affinities of both factor Xa and factor Va have been reported to decrease proportionally with the phosphatidylserine content of the membrane below 20% phosphatidylserine (Nelsestuen & Broderius, 1977; Bloom et al., 1979). It is interesting to note that the K_d becomes independent of the phospholipid concentration at a low phosphatidylserine content of the vesicle (Table III).

Thus the K_d that describes the interaction between factor Xa and factor Va in the presence of phospholipid is an apparent K_d depending on both the phospholipid concentration and the composition (phosphatidylserine content) of the phospholipid vesicles.

The precise manner by which phospholipid promotes complex formation is not known. Two models have been postulated: (1) Phospholipid binding increases the local concentrations of the reactants, and as a result the equilibrium of complex formation shifts into the direction of association (Rosing et al., 1980). (2) The interaction between the reactants is direct in the three-dimensional sense. The increased affinity would result from additive effects of protein–phospholipid and protein–protein interactions (Nelsestuen, 1978).

Thus, the formation of the factor Xa–Va complex will depend on the factor Va concentration on the phospholipid surface (model 1) or on the concentration of factor Va in the bulk solution (model 2). If one is given the published binding parameters (Bloom et al., 1979) and the amount of factor Va added required to achieve half-saturation of phospholipid-bound factor Xa (apparent K_d value), the concentration of phospholipid-bound factor Va and the concentration of factor Va in bulk solution can be calculated (Table V). The dependency of the apparent K_d of the phospholipid concentration and phosphatidylserine content of the vesicle can be rationalized quantitatively in the context of both models.

Model 1. The affinity of factor Va for vesicles increases up to about 20% phosphatidylserine. In order to obtain a factor Va concentration of 1–2 μmol of factor Va/mol of phospholipid at the phospholipid surface required to achieve half-saturation of phospholipid-bound factor Xa, it is necessary to add less factor Va with increasing phosphatidylserine content. Increased affinity also implies that the phospholipid concentration might become inhibitory. The amount of added factor Va has to increase with the increase in phospholipid concentration to attain 1–2 μmol of factor Va/mol of phospholipid at the surface.

Model 2. An increase in phospholipid concentration will competitively remove factor Va from interaction with the factor Xa–phospholipid site, depending on the affinity of factor Va for the phospholipid surface. Consequently, the amount of added factor Va has to increase with increase in phospholipid concentration to attain a factor Va concentration in the bulk solution to achieve half-saturation of phospholipid-bound factor Xa.

The combined results of this study support the notion that the enzymatic component of the prothrombin converting complex consists of an equimolar phospholipid-bound complex of factor Xa and factor Va. In addition, the "quality" of the phospholipid surface (phosphatidylserine content) upon which the factor Xa–Va complex is formed can act as a regulator of the catalytic activity of the prothrombinase complex. Therefore, our data have to be taken into consideration in a discussion about the physiological significance of negatively charged phospholipids exposed on the outer surface of thrombin/collagen-activated platelets (Bevers et al., 1982).

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Conformational Analysis of Small Disulfide Loops. Spectroscopic and Theoretical Studies on a Synthetic Cyclic Tetrapeptide Containing Cystine†

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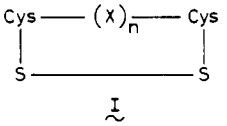
ABSTRACT: The conformational analysis of the synthetic peptide Boc-Cys-Pro-Val-Cys-NHMe has been carried out, (S–S) as a model for small disulfide loops, in biologically active polypeptides. ^{1}H NMR studies (270 MHz) establish that the Val(3) and Cys(4) NH groups are solvent shielded, while ^{13}C studies establish an all-trans peptide backbone. Circular dichroism and Raman spectroscopy provide evidence for a right-handed twist of the disulfide bond. Analysis of the vicinal ($J_{\alpha\beta}$) coupling constants for the two Cys residues establishes that $\chi^1 \sim \pm 60°$ for Cys(4), while some flexibility is suggested at Cys(1). Conformational energy calculations, imposing intramolecular hydrogen bonding constraints, favor a β-turn (type I) structure with Pro(2)-Val(3) as the corner residues. Theoretical and spectroscopic results are consistent with the presence of a transannular 4 → 1 hydrogen bond between Cys(1) CO and Cys(4) NH groups, with the Val NH being sterically shielded from the solvent environment.

The formation of disulfide bonds between cysteine residues located at different points in a polypeptide chain leads to the generation of compact structures. When the number of intervening amino acids separating the two Cys residues is large, the loops formed can have considerable conformational flexibility. However, if the number of spacer amino acids is small, then the reduction in ring size introduces stereochemical constraints. The formation of such conformationally well-defined structural units in proteins, hormones, and polypeptide toxins may have some importance in recognition processes like receptor interactions. Small ring peptide disulfides (I) ($n \leq$



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4) are frequently observed in hormones, e.g., oxytocin and vasopressin (n = 4, 20-membered ring) (Hruby, 1981), insulin (n = 4) (Blundell et al., 1972), and snake toxins, e.g., long neurotoxin *Siamensis* (n = 4 and n = 3, 17-membered ring) (Karlson et al., 1971). An important example of an n = 2 loop (14-membered ring) is the -Cys-Gly-Pro-Cys- segment, which constitutes the active site of the redox protein thioredoxin (Holmgren, 1981). While considerable interest has been centered on the conformation of oxytocin, vasopressin, and their analogues (Urry & Walter, 1971; Fischman et al., 1980; Tu et al., 1979; Wyssbrod et al., 1977, 1979; Hruby et al., 1979a,b; Mosberg et al., 1981), relatively little information is available on smaller cyclic peptide disulfides.

The 8-membered ring (n = 0) -Cys-Cys- (S–S) fragment in malformin A (Ptak, 1973; Tonelli, 1978) and the 17-membered ring (n = 3) Cys-Ser-Gly-Gly-Cys (S–S), found in pepsin (Klis & Siemion, 1978), have been studied experimentally. Structural studies on the model peptides cyclo(L-cystine) (Donzel et al., 1972; Varughese et al., 1981) and cyclo(cysteinylcysteine) (Capasso et al., 1977) have also been reported. As part of a program to explore the conformational characteristics of cyclic peptide disulfides, we describe in this report the synthesis and

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