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Binding of human blood-coagulation Factors IXa and X to phospholipid membranes

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A simple centrifugation technique has been developed to study the interaction of human coagulation Factors IXa and X with phospholipid membranes. In the presence of Ca^{2+}, equimolar phosphatidylserine/phosphatidylcholine membranes form tight complexes with Factor X ($K_D = 2.8 \times 10^{-8}$ M); the K_D is independent of the phospholipid concentration. Binding sites are available for about 2 mmol of Factor X/mol of phospholipid. Factor IXa has a slightly higher affinity for the phospholipid membrane ($K_D = 1.2 \times 10^{-8}$ M), and competes with Factor X for binding. The experimentally observed competition between Factor X and Factor IXa is in agreement with a model that describes the binding of two distinct ligands to a single class of independent binding sites.

In the intrinsic pathway of blood coagulation, Factor X is activated by a complex consisting of the serine proteinase Factor IXa and the cofactors Factor VIII, Ca^{2+} and phospholipid (Davie *et al.*, 1979; Jackson & Nemerson, 1980). Of the proteins involved in this complex, Factor IXa and Factor X belong to the vitamin K-dependent coagulation factors, which contain γ-carboxyglutamic acid residues involved in Ca^{2+}-mediated protein–phospholipid interactions (Stenflo & Suttie, 1977; Nelsestuen *et al.*, 1978). Also, Factor VIII has been reported to bind to phospholipid, probably in a Ca^{2+}-independent manner (Andersson & Brown, 1981; Lajmanovich *et al.*, 1981). Generally, these data support the notion that phospholipid participates in the intrinsic activation of Factor X by providing a surface for complex-formation between Factor IXa, Factor VIII and Factor X (Zwaal, 1978; Jackson & Nemerson, 1980). More insight into this role of the phospholipid surface may be obtained by studying its effect on the rate of Factor Xa formation; for the interpretation of such studies, quantitative data are required that describe the interaction of all relevant coagulation factors with the phospholipid membrane under the conditions of the kinetic experiments. For the bovine system, kinetic studies have shown that phospholipid enhances the Factor X activation by decreasing the K_m of Factor X (van Dieijen *et al.*, 1981*a*). Although the binding of bovine Factor X to phospholipids has been studied in detail (Nelsestuen & Broderius, 1977; Bloom *et al.*, 1979; van Dieijen *et al.*, 1981*b*), such data are not available for the interaction of the other reactants with the phospholipid membrane.

Our investigations concern the intrinsic activation of Factor X using the purified human proteins. The present paper describes a simple technique to study the phospholipid-binding properties of human Factors IXa and X under the conditions of kinetic experiments. It is demonstrated that Factor IXa and Factor X have similar membrane affinities and compete for binding to the same sites on the phospholipid membrane. These data can be used for the interpretation of experiments concerning the Factor Xa formation under identical experimental conditions [see the following paper (Mertens & Bertina, 1984)].

Abbreviations used: NPGB, 4-nitrophenyl 4-guanidinobenzoate hydrochloride; PS, L-α-phosphatidyl-L-serine; PC, L-α-phosphatidylcholine; SDS, sodium dodecyl sulphate.

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Experimental

Materials

Heparin–Sepharose CL-6B and Sephadex G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Tris (TRIZMA base), Russell's-viper

(*Vipera russelli*) venom, L-α-phosphatidyl-L-serine (type P-6641) and L-α-phosphatidylcholine (type P-4139) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ovalbumin (five-times-recrystallized) was purchased from Koch–Light Laboratories, Colnbrook, Slough, Berks., U.K., and 4-nitrophenyl 4-guanidinobenzoate hydrochloride (NPGB) from BDH Chemicals, Poole, Dorset, U.K. Benzamidine hydrochloride was from Aldrich Europe, Beerse, Belgium. All other chemicals were analytical-grade products from Merck, Darmstadt, Germany.

Phospholipid vesicles. PS was a preparation from bovine brain, whereas PC was highly purified hydrogenated egg lecithin; both phospholipids were approx. 98% pure according to the supplier's estimate. Equimolar mixtures of PS and PC were prepared from stock solutions of the purified products in chloroform. After evaporation of the solvent in a stream of N_2, the lipid was dispersed in 100mM-NaCl/50mM-Tris/HCl, pH 7.5 (0.2ml/μmol of phospholipid) at 0°C for five periods of 2min with 5min cooling intervals, using a model B-12 sonifier (Branson, Danbury, CT, U.S.A.) adjusted to 30W output. After sonication, the lipid dispersion was centrifuged (30min at 20000*g*) to remove sonicator-probe particles and large multilamellar liposomes. The phospholipid recovery in the supernatant vesicle preparation varied between 80 and 95%. This preparation is referred to as 'PS/PC vesicles' through the present paper. Phospholipid concentrations were determined by phosphate analysis after $HClO_4$ combustion (Böttcher *et al.*, 1961).

Purified coagulation factors. Protein concentrations were determined by measuring the A_{280} after correcting for Rayleigh scattering at 320nm (Shapiro & Waugh, 1966); the absorption coefficients ($A^{1\%}_{1cm,280}$) used were 11.6 and 13.3 for human Factors X and IX respectively (DiScipio *et al.*, 1977). When amounts of coagulation factors are expressed in units, 1 unit refers to the amount of activity or antigen present in 1ml of pooled normal human plasma.

Human Factor X was purified according to a simplification of a previously described method (Mertens & Bertina, 1980). In this procedure, steps 4 and 5 of the original method are replaced by one single step, using a commercially available heparin–Sepharose (Pharmacia). Factor II is slightly retarded on this column, whereas Factors X and IX are eluted subsequently in a linear NaCl gradient (0–1M) in 3mM-$CaCl_2$/10mM-benzamidine/10mM-triethanolamine, pH 6.35. A minor high-M_r contaminant was removed by gel filtration on a column (2.5cm × 95cm) of Sephadex G-200 in 10mM-benzamidine/1mM-EDTA/1M-NaCl/50mM-Tris/HCl, pH 8.0. The final preparation was concentrated, dialysed against 100mM-NaCl/50mM-Tris/HCl, pH 7.5, and stored in 250μl aliquots at −20°C. The Factor X preparations were homogeneous as judged by SDS/polyacrylamide-gel electrophoresis (Mertens & Bertina, 1980). The specific activity was 120–160 units/mg, when Factor X was measured spectrophotometrically (Mertens & Bertina, 1982), by electroimmunoassay (Bertina *et al.*, 1981), and by its coagulant activity in a kaolin-activated partial-thromboplastin-time assay (Veltkamp *et al.*, 1968) with a Factor X-depleted plasma (Bertina *et al.*, 1981) as substrate plasma.

Human Factor IXa was prepared by a previously described method (Mertens & Bertina, 1982). The required Factor IX had been isolated as the by-product of the simplified procedure for the isolation of Factor X; after the final gel filtration (Bertina & Veltkamp, 1978), the specific activity was about 250 units/mg as measured by coagulation assay (Veltkamp *et al.*, 1968), or about 500units/mg as estimated by electroimmunoassay (Bertina & van der Linden, 1977). After activation, more than 90% of the protein was active enzyme as determined by active-site titration (see below).

Binding of Factors IXa and X to phospholipid

Binding assay. For the measurement of the binding of Factor IXa or Factor X to PS/PC vesicles in the presence of Ca^{2+} (10mM), these components were incubated in 100mM-NaCl/50mM-Tris/HCl buffer, pH 7.5 containing ovalbumin (0.2mg/ml), at 37°C in conical polypropylene 500μl cups (Greiner Labortechnik, Nürtingen, Germany). The ovalbumin, phospholipid and Ca^{2+} were preincubated for 10min in 60% of the final volume, before the addition of Factor IXa and/or Factor X; the final volume was 400μl. The complete mixture was incubated at 37°C for 5min, and centrifuged (10min at 8000*g*) in a type-5412 microcentrifuge (Eppendorf, Hamburg, Germany). Immediately after centrifugation, samples from the supernatants were drawn for the quantification of non-bound Factor X or Factor IXa. At this stage, the temperature of the incubation mixture had never decreased more than 3°C.

Spectrophotometric assays for Factor X and Factor IXa. Factor X was assayed as described previously (Mertens & Bertina, 1982). Factor IXa was quantified by its Factor X-activating activity in the presence of 10mM-$CaCl_2$, 180μM-PS/PC vesicles and 0.4μM-Factor X. After measurement of the rate of Factor Xa formation, the concentration of Factor IXa was determined by intrapolation on a calibration curve relating rate of Factor Xa formation to Factor IXa concentration obtained by

using a reference Factor IXa preparation; details of this method are given in the following paper (Mertens & Bertina, 1984). In order to express the results in molar terms, the spectrophotometric assays were calibrated with active-site-titrated reference preparations (see below). The Factor X concentration in normal human plasma (1 unit/ml) was found to be 0.10 μM.

Calculation of binding parameters. After determining the molar concentrations of non-bound Factor X or Factor IXa, the bound concentrations were obtained by subtracting non-bound from total concentrations. Binding parameters were calculated from plots of free and bound concentrations as described by Scatchard (1949).

The dissociation constant of Factor IXa was also obtained indirectly, by measuring the competitive effect of Factor IXa on the Factor X–phospholipid interaction. The experimental data for Factor X binding were fitted to a model of two ligands that compete for a single class of binding sites, assuming that interaction between ligands can be neglected, and that an equal number of binding sites is available for Factor X and for Factor IXa (cf. Fig. 2 below). The binding of Factor X and Factor IXa to PS/PC membranes then is described by two equilibria:

$$X + P \rightleftharpoons X\text{–}P \qquad (1a)$$

and

$$IXa + P \rightleftharpoons IXa\text{–}P \qquad (1b)$$

where P represents one independent binding site on the PS/PC membrane. The dissociation constants of the complexes are given by:

$$K_1 = \frac{[X][P]}{[X\text{–}P]} \qquad (2a)$$

and

$$K_2 = \frac{[IXa][P]}{[IXa\text{–}P]} \qquad (2b)$$

The total concentration of binding sites, $[P]_0$, is the sum of the free and occupied binding sites:

$$[P]_0 = [P] + [X\text{–}P] + [IXa\text{–}P] \qquad (3)$$

The concentration of bound Factor IXa, [IXa–P], is the difference between $[IXa]_0$ and [IXa] (the total and free Factor IXa concentrations respectively); thus eqn. (2b) can be rewritten as:

$$[IXa\text{–}P] = \frac{[P] \cdot [IXa]_0}{K_2 + [P]} \qquad (4)$$

By substituting eqns. (2) and (4) in eqn. (3), the following expression for K_2 can be derived:

$$K_2 = K_1 \cdot R\left(\frac{[IXa]_0}{[P]_0 - K_1 \cdot R - [X\text{–}P]} - 1\right) \qquad (5)$$

where

$$R = \frac{[X\text{–}P]}{[X]}$$

By use of this equation, K_2 can be calculated at various values for $[IXa]_0$ and $[P]_0$, using K_1 and the experimentally established values for the concentration of free Factor X, [X], and bound Factor X, [X–P].

Other methods

Molar concentrations of human Factor X and Factor IXa were determined by active-site titration as described for the corresponding bovine proteins by Smith (1973) and Byrne *et al.* (1980). Titrations were performed at 30°C in 1 ml of 0.1 M-NaCl/0.1 M-Veronal, pH 8.3, containing $CaCl_2$ (5 mM), Factor IXa or Factor X (20–50 μg), glycerol (5%, v/v) and NPGB (10–100 μM). Factor IXa titrations were initiated by the addition of NPGB, whereas Factor X titrations were started by the addition of $CaCl_2$ to reaction mixtures containing Factor X and 7 μg of the purified Factor X-activating proteinase from Russell's-viper venom (Kisiel *et al.*, 1976). The extent of the pre-steady-state *p*-nitrophenol production, π (Bender *et al.*, 1966), was determined from A_{410} recordings obtained by using a type-PM 6 spectrophotometer (Zeiss, Oberkochen, Germany) equipped with a Servogor RE 511 recorder (Goerz, Vienna, Austria). The concentrations of Factor IXa or Factor X were calculated from the maximal obtainable value of π, as determined from plots of $1/\sqrt{\pi}$ versus 1/[NPGB] (Bender *et al.*, 1966), using ε_{410} 16595 $M^{-1} \cdot cm^{-1}$ for *p*-nitrophenol (Bender & Nakamura, 1962).

The rate of Factor Xa formation in mixtures containing Factor X, Factor IXa, Ca^{2+} and phospholipid was determined as described in the following paper (Mertens & Bertina, 1984).

The aggregation of PS/PC vesicles was measured by recording the turbidity (A_{405}) of phospholipid suspensions after the addition of Ca^{2+} (Chong & Colbow, 1976; Ohki *et al.*, 1982).

Results

Technique to study protein–phospholipid binding

The present method was developed to enable studies of the binding of Factors IXa and X to phospholipid membranes that contain 50 mol %

PS at Ca^{2+} concentrations of 10mM. These experimental conditions were the same as those used in kinetic studies concerning the activation of Factor X by Factor IXa [see the following paper, Mertens & Bertina, (1984)]. The technique is based on the tendency of PS/PC vesicles to aggregate in the presence of Ca^{2+}; after centrifugation of mixtures of PS/PC vesicles and Ca^{2+}, more than 97% of the phospholipid was recovered as a compact pellet, whereas no significant phospholipid sedimentation (<5%) occurred in the absence of Ca^{2+}. The minimal Ca^{2+} concentration required for complete sedimentation was about 3mM. The Ca^{2+}-induced sedimentation was independent of the presence of ovalbumin (0.2mg/ml), Factor X (0.4μM) or of the phospholipid concentration (tested between 0.02 and 1mM).

The use of aggregated PS/PC vesicles for binding studies requires stability of the phospholipid membrane during the experiments. As Fig. 1(*a*) shows, the addition of Ca^{2+} to PS/PC vesicles was followed by a rapid increase of the turbidity (A_{405}) of the incubation mixture. Except for dilution effects, the subsequent addition of Factor X and Factor IXa had no further effect on the turbidity. This suggests that aggregated PS/PC vesicles form a surface that is stable between 5 and at least 30min after the Ca^{2+} addition. In agreement with this observation, variation of the Ca^{2+}/phospholipid preincubation time between 5 and 30min had no effect on the Factor X concentration in the supernatant obtained after centrifugation (Fig. 1*b*), nor on the initial rate of Factor Xa formation (Fig. 1*c*) under conditions where the amount of available phospholipid is a rate-limiting factor (see Mertens & Bertina, 1984). On the basis of these data, the experimental scheme for the binding studies included (*a*) preincubation of Ca^{2+} and PS/PC vesicles for 10min, (*b*) incubation of Factor X and/or Factor IXa with the stable phospholipid surface, (*c*) centrifugation and (*d*) analysis of the supernatant for non-bound proteins (see the Experimental section for details).

Control experiments showed that the inclusion of ovalbumin in the incubation mixtures was essential in order to prevent non-specific adsorption of coagulation factors to the polypropylene centrifugation cups. With this precaution, no sedimentation of Factor X or Factor IXa occurred in the absence of phospholipids; furthermore, all added Factor IXa or Factor X was recovered in incubation mixtures that had been homogenized after centrifugation. The fraction of Factor X bound to preaggregated PS/PC vesicles was constant when the incubation time preceding the centrifugation was varied beween 2 and 30min; this implies that binding equilibrium was reached within 2min. It was concluded from these controls that the amounts of phospholipid-bound factors can be calculated by subtracting the amounts present in the supernatant from those totally added, and that these free and bound concentrations can be used for the calculation of equilibrium binding constants.

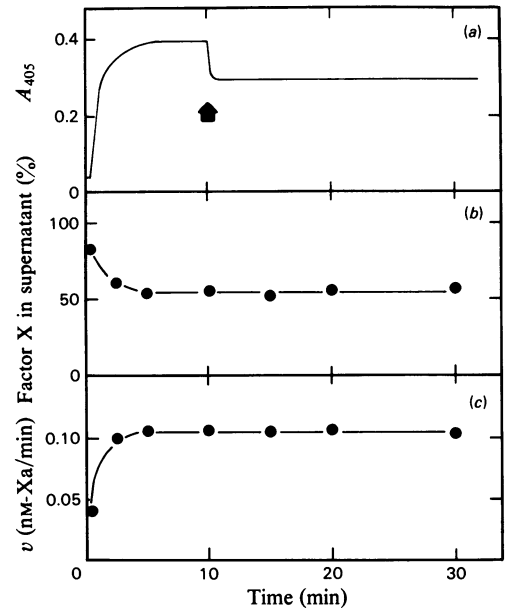

Fig. 1. *Stability of PS/PC membranes in the presence of Ca^{2+}*

PS/PC vesicles (100μM) were incubated in the presence of $CaCl_2$ (10mM), ovalbumin (0.2mg/ml), Factor X (0.4μM) and Factor IXa (3.6nM) in 100mM-NaCl/50mM-Tris/HCl, pH7.5, at 37°C; ovalbumin, phospholipid and Ca^{2+} were preincubated in 60% of the final volume before the addition of Factor IXa and Factor X (see the Experimental section). (*a*) Registration of turbidity (A_{405}) after the addition of Ca^{2+} to the PS/PC vesicles; after preincubation for 10min (see arrow), the mixture was completed by the addition of Factors IXa and X. (*b*) The effect of the Ca^{2+}/phospholipid preincubation time on the percentage of Factor X recovered in the supernatant after centrifugation of the incubation mixture. (*c*) The effect of the Ca^{2+}/phospholipid preincubation time on the initial rate of Factor Xa formation (v).

Binding of Factor X and Factor IXa to phospholipids

The above-described centrifugation technique was used to study the binding of human Factors IXa and X to PS/PC membranes in the presence of Ca^{2+}. Fig. 2 shows the results in the form of Scatchard plots. For Factor X, K_D was found to be 2.8×10^{-8}M. The same K_D value was found at three phospholipid concentrations. The number of

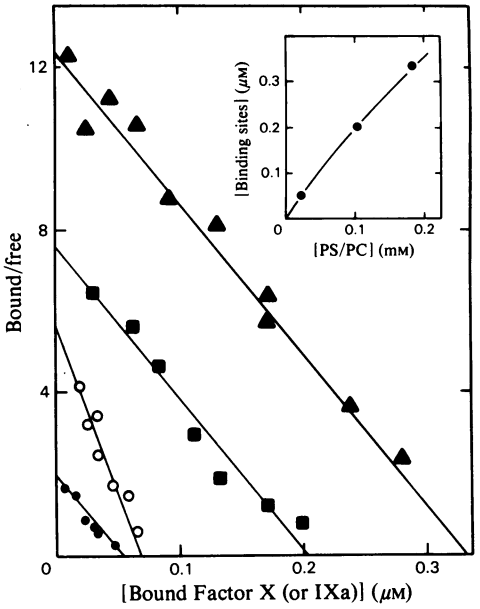

Fig. 2. *Scatchard plots for the binding of Factor X and Factor IXa to PS/PC membranes*
Various amounts of Factor X were incubated with 20 (●), 100 (■) or 180 (▲) μM-PS/PC at 37°C in 10mM-$CaCl_2$/100mM-NaCl/50mM-Tris/HCl, pH7.5, containing ovalbumin (0.2mg/ml). (○) Various amounts of Factor IXa were incubated with PS/PC vesicles (20 μM) under the same conditions as for Factor X. The amounts of free and bound Factor X or Factor IXa were determined as described in the Experimental section. The inset shows a replot of the number of Factor X-binding sites against the PS/PC concentration.

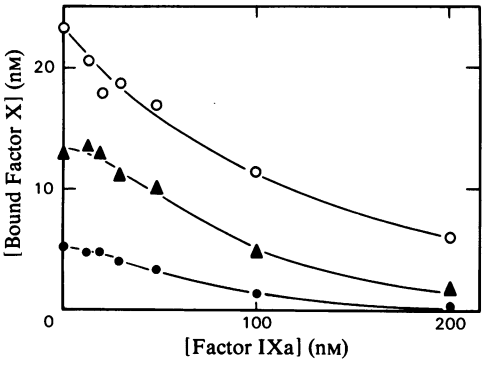

Fig. 3. *Inhibition of the Factor X–phospholipid interaction by Factor IXa*
Various amounts of Factor IXa were incubated with PS/PC vesicles (20 μM) in the presence of 8.3 (●), 23.7 (▲) and 48.0 (○) nM-Factor X at 37°C in 10mM-$CaCl_2$/100mM-NaCl/50mM-Tris/HCl, pH7.5, containing ovalbumin (0.2mg/ml). Factor X was incubated with preaggregated PS/PC vesicles for 8min in 94% of the final volume, before the addition of Factor IXa; after a further incubation for 8min, the amounts of phospholipid-bound Factor X were determined as described in the Experimental section.

available Factor X-binding sites depended almost linearly on the PS/PC concentration (see Fig. 2, inset): about 2mmol of Factor X could be bound per mol of phospholipid. The linearity of the Scatchard plots of Fig. 2 indicates that the Factor X-binding sites are identical and independent. Factor IXa was found to have a slightly higher affinity for PS/PC membranes ($K_D = 1.2 \times 10^{-8}$ M) than Factor X. At 20 μM-phospholipid, a similar number of binding sites was available for Factor X and Factor IXa (see Fig. 2).

Competition of Factor IXa and Factor X for binding to phospholipid

The sites on PS/PC membranes that are involved in the binding of Factor IXa and Factor X may be the same. In that case, both these factors should compete for binding, since the K_D values for their interactions with PS/PC membranes are similar (see Fig. 2). This was tested by studying the effect of various concentrations of Factor IXa on the Factor X–phospholipid interaction. The results are shown in Fig. 3. When the total Factor X concentration was 8.3nM, Factor IXa readily displaced all bound Factor X from the phospholipid surface. This demonstrates that the phospholipid binding of Factor X is completely reversible. In the experiments of Fig. 3, Factor IXa was added after Factor X; reversal of these additions had no effect on the results. Fig. 3 further indicates that, when the Factor X concentration was increased, more Factor IXa was needed for complete Factor X displacement. Qualitatively, these observations are in agreement with competition of Factor IXa and Factor X for binding to the same sites on the PS/PC membrane. The simplest quantitative description of such a system is given by a model of two distinct ligands that compete for a single class of independent binding sites. To test the validity of this model, the data of Fig. 3 were fitted to an equation [see eqn. (5) in the Experimental section] that was derived to calculate the dissociation constant of the competitor, Factor IXa, on the basis of binding data for the second ligand, Factor X. By this method, the dissociation constant of Factor IXa was calculated to be $1.7(\pm 1.0) \times 10^{-8}$ M (mean ± S.D., $n = 16$). Within the experimental error, this value is in agreement with the K_D of 1.2×10^{-8} M that was found for Factor IXa in the absence of Factor X (see Fig. 2). It was concluded that the relatively simple model provides an

adequate description of the competitive binding of Factor IXa and Factor X to PS/PC membranes.

Discussion

The aim of the present study was to obtain binding parameters for the interaction of human Factors IXa and X with phospholipid membranes. These parameters were to be used for the interpretation of kinetic data concerning the contribution of phospholipid to the activation of Factor X by Factor IXa (see Mertens & Bertina, 1984). Therefore it was essential that binding and kinetic studies could be performed under identical experimental conditions.

In most current methods of studying the binding of coagulation factors to phospholipid membranes, the experimental approach and/or conditions differ markedly from those of a kinetic experiment. This applies for instance to methods based on gel-filtration techniques (Dombrose *et al.*, 1979) or on adsorption of radiolabelled proteins to phospholipid monolayers (Lecompte *et al.*, 1980). In this regard, also the technique of 90° light-scattering (Nelsestuen & Lim, 1977) has certain limitations: although this method has been applied successfully to various isolated coagulation factors (Nelsestuen & Broderius, 1977; Nelsestuen *et al.*, 1978; Bloom *et al.*, 1979), it is less suitable to evaluate binding in mixtures of coagulation factors; furthermore, its experimental conditions are restricted to low Ca^{2+} concentrations and membranes of low PS content (Nelsestuen & Lim, 1977). To study the phospholipid binding of bovine Factor X under conditions of kinetic experiments, van Dieijen *et al.* (1981*b*) have developed a method that is based on the observation that Russell's-viper venom cannot activate phospholipid-bound Factor X. In preliminary experiments, we have confirmed the validity of this technique with bovine Factor X; however, the method failed to detect binding of human Factor X under the same conditions.

The technique described in the present paper is based on the Ca^{2+}-induced aggregation of PS/PC vesicles, which enables separation of free and phospholipid-bound proteins by centrifugation. The major advantage of this method is that it can be applied directly to incubation mixtures of kinetic experiments. A disadvantage might seem that aggregated phospholipid vesicles provide a less-characterized surface than do unilamellar vesicles. However, it should be realized that structural changes of PS-containing vesicles on the addition of Ca^{2+} are inevitable; these may include aggregation of phospholipid vesicles (Papahadjopoulos *et al.*, 1975; Day *et al.*, 1977; Ohki *et al.*, 1982) and lateral phase separation resulting in PC- and PS-rich domains (Tokutomi *et al.*, 1979; Hui *et al.*, 1983). Just the same conditions (Ca^{2+} concentration, PS content) that are associated with the formation of soluble aggregates also favour the activation of Factor X (see Mertens & Bertina, 1984). The aggregated PS/PC vesicles were found to provide a stable phospholipid surface (Fig. 1). We therefore concluded that the present technique permits binding studies under conditions that are particularly relevant to Factor Xa formation.

The K_D for the interaction between human Factor X and PS/PC membranes was found to be 2.8×10^{-8} M (Fig. 2). This value agrees well with the K_D of 4×10^{-8} M that was reported for the binding of bovine Factor X to membranes of the same PS content (van Dieijen *et al.*, 1981*b*). Other investigators have reported higher K_D values for bovine Factor X (Nelsestuen & Broderius, 1977; Bloom *et al.*, 1979). This discrepancy is probably due to differences in experimental conditions: those studies employed phospholipid vesicles of lower PS content, which is known to result in higher K_D values (van Dieijen *et al.*, 1981*b*); also, other conditions (Ca^{2+} concentration, pH, temperature) may contribute to the differences. Human Factor IXa has a slightly higher affinity for PS/PC membranes ($K_D = 1.2 \times 10^{-8}$ M) than does Factor X (Fig. 2). This seems to disagree with data of Nelsestuen *et al.* (1978), who reported that bovine Factors IX and IXa bind less tightly than bovine Factor X. However, the bovine Factor IXa used in those studies was a non-physiological form of Factor IXa that still contained the activation peptide (Lindquist *et al.*, 1978). Together with various experimental differences (see above), this hinders a direct comparison of the dissociation constants of bovine and human Factor IXa.

Aggregated PS/PC vesicles provide a similar number of binding sites for Factor X and Factor IXa (Fig. 2). The number of available sites (2 mmol of Factor X per mol of phospholipid) is about 30% of that found by van Dieijen *et al.* (1981*b*) for vesicles of the same (50 mol%) PS content. Vesicles containing about 20 mol% PS have been reported to possess five to ten times more Factor X-binding sites (Nelsestuen & Broderius, 1977; Bloom *et al.*, 1979). The relatively low number of sites in our study may be explained by the pre-aggregation of the PS/PC vesicles, which served to provide a stable surface in the presence of Ca^{2+}, and to prevent trapping of coagulation factors within aggregates (see Fig. 1). Aggregation is probably associated with a decrease in the number of available protein-binding sites (see also van Dieijen *et al.*, 1981*b*).

Factor IXa and Factor X compete for binding to PS/PC membranes (Fig. 3). A similar competition between two vitamin K-dependent proteins has been observed by van Dieijen *et al.* (1981*b*), who

reported that bovine Factors X and II compete for binding sites on PS/PC vesicles. Whether the competition between Factors IXa and X also occurs during blood coagulation *in vivo* is not clear. Physiological membranes may differ from PS/PC membranes by their PS content, which is known to affect the K_D of bovine Factor X considerably (Nelsestuen & Broderius, 1977; van Dieijen *et al.*, 1981*b*), or by exposing specific receptors that are not present on model membranes. In this regard it is of interest that bovine Factors IX and X bind to endothelial cells with K_D values that are similar to those obtained in our study, but do not compete for binding to the same sites on the endothelial-cell membrane (Heimark & Schwartz, 1983).

The competitive binding of Factors IXa and X to PS/PC membranes complicates kinetic studies concerning the role of phospholipid in the activation of Factor X by Factor IXa; for instance, systematic variation of the Factor X concentration will not only affect the distribution of the substrate between solution and phospholipid surface, but will also influence the phospholipid binding of the enzyme, Factor IXa. The experimentally observed competition between Factor X and Factor IXa (Fig. 3) can be described adequately by a model of two distinct ligands that compete for a single class of binding sites. This model provides a method to predict the concentrations of free and phospholipid-bound Factors IXa and X in kinetic experiments by using the binding parameters obtained in the present study. This approach to the interpretation of kinetic studies is described in the following paper (Mertens & Bertina, 1984).

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References

Andersson, L.-O. & Brown, J. E. (1981) *Biochem. J.* **200**, 161–167

Bender, M. L. & Nakamura, K. (1962) *J. Am. Chem. Soc.* **84**, 2577–2582

Bender, M. L., Begué-Cantón, M. L., Blakely, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kézdy, F. J., Killheffer, J. V., Marshall, T. H., Miller, C. G., Roeske, R. W. & Stoops, J. K. (1966) *J. Am. Chem. Soc.* **88**, 5890–5913

Bertina, R. M. & van der Linden, I. K (1977) *Clin. Chim. Acta* **77**, 275–286

Bertina, R. M. & Veltkamp, J. J. (1978) *Thromb. Haemostasis* **40**, 335–349

Bertina, R. M., Westhoek-Kuipers, M. E. J. & Alderkamp, G. H. J. (1981) *Thromb. Haemostasis* **45**, 237–241

Bloom, J. W., Nesheim, M. E. & Mann, K. G. (1979) *Biochemistry* **18**, 4419–4425

Böttcher, C. J. F., van Gent, C. M. & Pries, C. (1961) *Anal. Chim. Acta* **24**, 203–204

Byrne, R., Link, R. P. & Castellino, F. J. (1980) *J. Biol. Chem.* **225**, 5336–5341

Chong, C. S. & Colbow, K. (1976) *Biochim. Biophys. Acta* **436**, 260–282

Davie, E. W., Fujikawa, K., Kurachi, K. & Kisiel, W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **48**, 277–318

Day, E. P., Ho, J. T., Kunze, R. K. & Sun, S. T. (1977) *Biochim. Biophys. Acta* **470**, 503–508

DiScipio, R. G., Hermodson, M. A., Yates, S. G. & Davie, E. W. (1977) *Biochemistry* **16**, 698–706

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

Heimark, R. L. & Schwartz, S. M. (1983) *Biochem. Biophys. Res. Commun.* **111**, 723–731

Hui, S. W., Boni, L. T., Stewart, T. P. & Isac, T. (1983) *Biochemistry* **22**, 3511–3516

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

Kisiel, W., Hermodson, M. A. & Davie, E. W. (1976) *Biochemistry* **15**, 4901–4906

Lajmanovich, A., Hudry-Clergeon, G., Freyssinet, J. M. & Marguerie, G. (1981) *Biochim. Biophys. Acta* **678**, 132–136

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

Lindquist, P. A., Fujikawa, K. & Davie, E. W. (1978) *J. Biol. Chem.* **253**, 1902–1909

Mertens, K. & Bertina, R. M. (1980) *Biochem. J.* **185**, 647–658

Mertens, K. & Bertina, R. M. (1982) *Thromb. Haemostasis* **47**, 96–100

Mertens, K. & Bertina, R. M. (1984) *Biochem. J.* **223**, 607–615

Nelsestuen, G. L. & Broderius, M. (1977) *Biochemistry* **16**, 4172–4177

Nelsestuen, G. L. & Lim, T. K. (1977) *Biochemistry* **16**, 4164–4171

Nelsestuen, G. L., Kisiel, W. & DiScipio, R. G. (1978) *Biochemistry* **17**, 2134–2138

Ohki, S., Düzgünes, N. & Leonards, K. (1982) *Biochemistry* **21**, 2127–2133

Papahadjopoulos, D., Vail, W. J., Jacobson, K. & Poste, G. (1975) *Biochim. Biophys. Acta* **394**, 483–491

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672

Shapiro, S. S. & Waugh, D. F. (1966) *Thromb. Diath. Haemorrh.* **16**, 469–490

Smith, R. L. (1973) *J. Biol. Chem.* **248**, 2418–2423

Stenflo, J. & Suttie, J. W. (1977) *Annu. Rev. Biochem.* **46**, 157–172

Tokutomi, S., Eguchi, G. & Ohnishi, S. I. (1979) *Biochim. Biophys. Acta* **552**, 78–88

van Dieijen, G., Tans, G., Rosing, J. & Hemker, H. C. (1981*a*) *J. Biol. Chem.* **256**, 3433–3442

van Dieijen, G., Tans, G., van Rijn, J., Zwaal, R. F. A. & Rosing, J. (1981*b*) *Biochemistry* **20**, 7096–7101

Veltkamp, J. J., Drion, E. F. & Loeliger, E. A. (1968) *Thromb. Diath. Haemorrh.* **19**, 279–303

Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta* **515**, 163–205