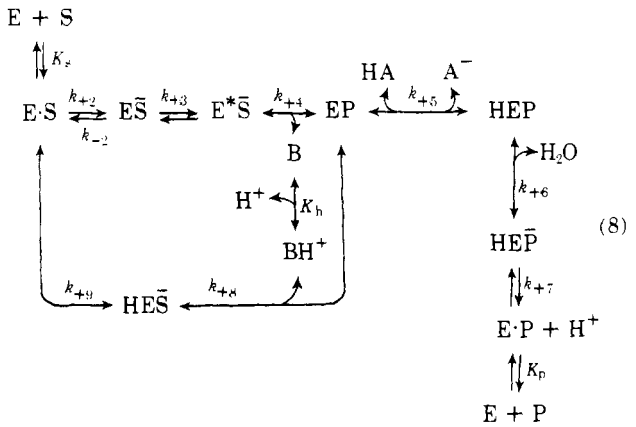
in the deacylation before the nucleophilic attack of water, and β-alanine reacts in its return reaction in the $-NH_3^+$ form.



(8)

The reaction mechanism of eq 8 gives rise to the steady state reaction velocity eq 9. The substrate binding reaction, the product dissociation reaction, as well as reaction $H^+ + B \rightleftharpoons BH^+$ are assumed to be in equilibria, and their reaction velocity constants are omitted. The reverse reaction velocity constants of reactions 3, 4, 6, 7, 8, and 9 are omitted as well, because the forward reactions 4, 7, and 9 are assumed to be very fast, and the above reverse reactions would then become insignificant.

$$v = \frac{e}{c_1 B + c_2} \tag{9}$$

where e = total enzyme concentration

$$c_1 = \frac{k_{+8}}{k_{+5}[HA]}\left(1 + \frac{k_{-5}[A]}{k_{+6}}\right)\left(\frac{1}{k_{+2}} + \frac{1}{k_{+3}} + \frac{1}{k_{+4}} + \frac{1}{k_{+9}} + \frac{k_{-2}}{k_{+2}k_{+3}}\right)$$

$$c_2 = \frac{1}{k_{+2}} + \frac{1}{k_{+3}} + \frac{1}{k_{+4}} + \frac{1}{k_{+6}} + \frac{1}{k_{+7}} + \frac{k_{-2}}{k_{+2}k_{+3}} + \frac{1}{k_{+5}[HA]} + \frac{k_{-5}[A]}{k_{+6}k_{+5}[HA]}$$

Equation 9 is of the same form as eq 2 and thus the progress curve is parabolic.

Figure 10 shows computed curves derived from the mechanism of eq 8. The curves are quite similar to the experimental curves in Figure 8a. Although the real reaction velocity constants are not known, Figure 10 shows that it is possible to estimate the reaction velocity constants so that eq 9 explains the experimental results.

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Binding of Bovine Coagulation Factor X_a to Platelets†

Björn Dahlbäck and Johan Stenflo*

ABSTRACT: The binding of highly purified bovine coagulation factor X_a to washed bovine platelets was studied. ^{125}I-labeled factor X_a underwent binding to a platelet receptor that became accessible only after induction of the platelet release reaction by thrombin or by the calcium ionophore A 23187. The zymogen factor X did not bind to platelets. The factor X_a binding was saturable, reversible, and correlated with the rate of thrombin formation. The number of factor X_a binding sites per platelet was 290–420 and the apparent association constant was estimated to be 2.8×10^9 to 1.0×10^{10} M^{-1}. Diisopropyl fluorophosphate–factor X_a bound to the same platelet receptor as factor X_a indicating that limited proteolysis of a receptor protein was not required for binding. The rate of factor X_a binding was rapid (2.1×10^6 to 2.9×10^6 M^{-1} s^{-1}) and similar to that previously found for the rate of binding of polypeptide hormones to their receptors. Displacement of factor X_a from the platelet receptor by diisopropyl fluorophosphate–factor X_a effectively blocked thrombin formation. Low concentrations of factor X_a catalyze prothrombin activation more effectively in the presence of platelets than in the presence of phospholipid and factor V.

Platelets have a central role in hemostasis (Weiss, 1975; Gordon, 1976). Subsequent to vascular injury, platelets adhere to subendothelial collagen and aggregate to each other forming a so called hemostatic plug. This process is accompanied by a release reaction, i.e., exocytosis of platelet granules with release of both high and low molecular weight components such as fibrinogen, Ca^{2+}, serotonin, and adenosine disphosphate. The aggregated platelets appear to provide a catalytic surface for localized activation of the plasma clotting factors. This

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0006-2960/78/0417-4938$01.00/0

process leads to fibrin formation and consolidation of the hemostatic plug.

The last step in the blood coagulation cascade (Davie & Fujikawa, 1975) is the conversion of prothrombin to thrombin by factor X_a. The rate of activation of prothrombin by factor X_a is, in the presence of calcium ions, greatly accelerated by phospholipid and factor V (Suttie & Jackson, 1977). Both factor X_a and prothrombin bind to factor V (Suttie & Jackson, 1977; Freeman et al., 1977) and to phospholipid vesicles (Suttie & Jackson, 1977; Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977; Lim et al., 1977; Dombrose et al., 1978). The binding to phospholipid requires presence of calcium ions (Suttie & Jackson, 1977; Dombrose et al., 1978). In vitro platelet lipid extracts stimulate blood clotting and platelet lipoproteins are even more active in this respect (Marcus et al., 1966). Information on the interaction of the vitamin K-dependent proteins with platelets or platelet subcellular structures has, however, been lacking. It was therefore an important step forward when Miletich et al. (1977) demonstrated that human factor X_a bound to platelets that had undergone the release reaction and catalyzed thrombin formation, whereas the zymogen, factor X, did not bind to the platelets (Miletich et al., 1977). The factor X_a receptor appeared to be a protein and had certain properties in common with factor V (Miletich et al., 1977).

The interaction between prothrombin and phospholipid vesicles has recently been studied in detail (Suttie & Jackson, 1977; Lim et al., 1977; Nelsestuen, 1976; Nelsestuen et al., 1976). Very little is known, however, about the physiologically important interaction between prothrombin and platelets. Tollefsen et al. (1975) reported that they could not demonstrate binding of prothrombin to platelets. Using an oil centrifugation technique that ensures rapid separation of platelet bound and free prothrombin (Miletich et al., 1977; Feinberg et al., 1974; Martin et al., 1976), we have also been unable to demonstrate binding of prothrombin (1 ng/mL to 0.25 mg/mL) to platelets both prior to and following induction of release. This may be due to a very rapid rate of dissociation of prothrombin from a platelet receptor. Other possibilities are that a prothrombin receptor must be proteolytically modified by factor X_a before actual binding of prothrombin can occur or that the factor X_a molecule itself is part of the prothrombin receptor. Before studying interaction between prothrombin and platelets we therefore wanted to characterize the factor X_a binding to bovine platelets in some detail. Bovine platelets were used in these experiments since bovine plasma is available in large amounts and since the vitamin K dependent proteins from bovine plasma are more easily purified and far better chemically characterized than the corresponding human proteins. This paper reports on the characterization of the factor X_a binding to bovine platelets.

Materials and Methods

Factor VII and X deficient plasma, Russell's viper venom, the taipan snake venom (*Oxyuranus scutellatus scutellatus*), Folch fraction III, rabbit brain cephalin, diisopropyl fluorophosphate (Dip-F[1]), phenylmethanesulfonyl fluoride $PhCH_2SO_2F$, and bovine serum albumin (Cohn fraction V) were from Sigma Chemical Co, St. Louis, Mo.; apiezon oil was from J.B. Biddle Co and di-*n*-butyl phthalate from the British Drug House, Poole, England. [^{14}C]Hydroxytryptamine binoxalate (44 μCi per μmol) was from New England Nuclear, Dreieichenhain, West Germany, and Na^{125}I, carrier free, from the Radiochemical Center, Amersham, England. The calcium ionophore A 23 187 (Lilly) was a kind gift from Dr Ingemar Lundkvist, University of Lund, Lund, Sweden. Millipore filters (RAW 025-00), 1.2-μm pore diameter, were from Millipore AB, Gothenburg, Sweden. Bovine fibrinogen (90% clottable) was from Miles Laboratories, Inc., Kankakee, Ill.

Bovine prothrombin was purified as described previously (Stenflo, 1976). It was dialyzed against 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.35, and stored at a protein concentration of 6.7 mg per mL at −70 °C. Prothrombin was quantified using an $E_{1cm}^{1\%}$ at 280 nm of 14.6 (Stenflo, 1972). Bovine thrombin was prepared by activation of bovine prothrombin with the taipan snake venom and purified by QAE-Sephadex chromatography (Owen & Jackson, 1973). It had a fibrinogen clotting activity of 620 NIH units per mg of protein. Highly purified bovine factor V was a generous gift from Dr. Peter Esnouf, Radcliff Infirmary, Oxford, England. The specific activity of factor V was given to be 80 units per mL per absorbance unit at 280 nm when the factor V activity of bovine plasma was defined to be 1 unit per mL. Incubation with thrombin did not further increase the factor V activity. A phospholipid suspension was prepared from Folch fraction III, dissolved to 50 mg per mL in chloroform:methanol (2:1), and evaporated to dryness. Tris-HCl, 0.02 M, 0.15 M NaCl, pH 7.35, was added to a final phospholipid concentration of 20 mg per mL buffer and the suspension sonicated in a Branson sonication bath under N_2 until transparent (40–50 min).

Factor X. Bovine factor X was purified to homogeneity as described previously (Stenflo, 1976). Activation of factor X was carried out with the factor X activator from Russell's viper venom (Schiffman et al., 1969) as described by Jesty & Nemerson (1976). The active enzyme was isolated by DEAE-Sephadex Chromatography, concentrated by Amicon ultrafiltratior using the UM 10 filter, dialyzed against 0.05 M Tris-HCl, 0.1 M NaCl, 50% (v/v) glycerol, pH 7.5, and stored at −20 °C. Factor X was quantified spectrophotometrically using an $E_{1cm}^{1\%}$ at 280 nm of 12.4 (Jackson, 1972).

The factor X_a preparations were initially homogeneous on agarose gel electrophoresis in 0.075 M barbital buffer, 2 mM EDTA, pH 8.6 (Johansson, 1972). After a few weeks at −20 °C a slight decrease in factor X_a activity was noticed and a narrow protein band more cathodal than factor X_a appeared. This cathodal protein (30% or less of the total protein), presumably a degradation product of the active protein, had no factor X_a activity. It gave a faint immunoprecipitate on crossed immunoelectrophoresis (Ganrot, 1972) with a monospecific antiserum against factor X (Stenflo, 1976) but did not bind to platelets. Two preparations of factor X_a were used. No difference in binding to platelets was observed between these two preparations.

Inactivation of factor X_a by DFP was performed essentially as described by Fujikawa et al. (1972). The factor X_a (0.26 mg/mL) was incubated with 5×10^{-3} M DFP in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.35, for 2 h at 37 °C. The pH did not fall below 7.0 during the incubation. Remaining active factor X_a, less than 0.1 %, was removed by filtration through a column (0.9 × 5 cm) of Sepharose 4B with covalently linked soybean trypsin inhibitor (Cuatrecasas, 1970). The eluate had no factor X_a activity. The DFP inactivated factor X_a was electrophoretically (Johansson, 1972) and immunochemically (Ganrot, 1972) indistinguishable from factor X_a. It was stored at +4 °C. Factor X_a (0.25 mg/mL) was also inactivated with $PhCH_2SO_2F$ by incubation with 2×10^{-3} M $PhCH_2SO_2F$ in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, overnight at 22 °C. After this treatment less than 0.1% factor X_a activity remained.

[1] Abbreviations used: Dip-F, diisopropyl fluorophosphate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

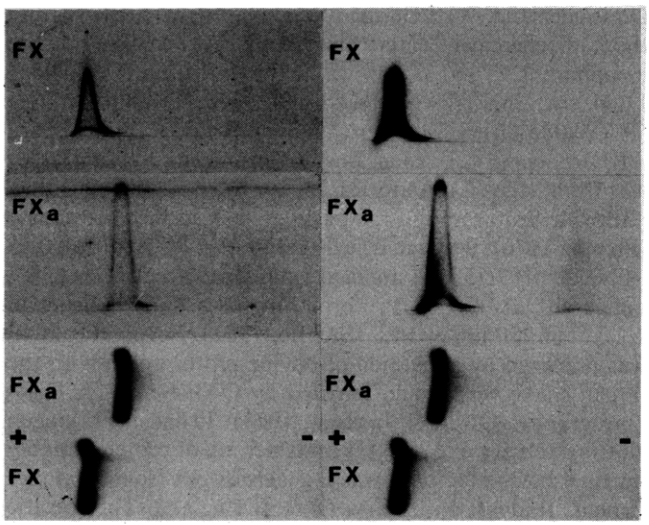

FIGURE 1: Patterns obtained by antigen–antibody crossed immunoelectrophoresis of labeled factor X and factor X_a in the presence of carrier. Protein staining to the left and autoradiography to the right. Below is shown the agarose gel electrophoretic patterns of unlabeled factor X and factor X_a. The agarose gel electrophoresis was run in 0.075 M barbital buffer, pH 8.6, containing 2 mM EDTA.

This preparation was used only in displacement experiments.

Clotting Assays. All assays were done in a Fibrometer coagulation timer (BBL). The method of Fenton & Fasco (1974) was used to determine thrombin activity. The assay was standardized with a thrombin standard, lot no. B-3, kindly supplied by Dr. D. L. Aronsen of the Department of Health, Education and Welfare, Bethesda, Md. Factor X_a activity was assayed according to Backman et al. (1958) using Cephalin without Russell's viper venom and factor VII and X deficient test plasma.

To study generation of thrombin in the presence of platelets, 0.8 mL of prothrombin, 0.17 mg/mL in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, containing 5 mg per mL of bovine serum albumin and 1 mg per mL of glucose was mixed with 0.1 mL of 25 mM $CaCl_2$ and 0.1 mL of platelets, 10^9 per mL, and incubated with factor X_a at 22 °C. Thrombin activity was measured in the fibrometer. In some experiments phospholipid and/or factor V was used instead of platelets.

Iodination Procedure. Factor X, factor X_a, Dip-F–factor X_a, and prothrombin were iodinated with sodium [^{125}I]iodide by a lactoperoxidase method essentially as described by Thorell & Johansson (1971). Free iodide was removed by gel filtration on a column of Sephadex G-25 (PD 10 Pharmacia Fine Chemicals), equilibrated with 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35. The protein containing fractions were pooled and stored in 100-μL aliquots at −70 °C after the addition of bovine serum albumin to 5 mg/mL. The specific radioactivities of ^{125}I-labeled factors X and X_a, Dip-F–factor X_a, and prothrombin were 800–1000 Ci/mmol and the mole ratios of iodide to factors X and X_a, DIP–factor X_a, and prothrombin were from 0.36 to 0.5. Iodinated factor X and prothrombin were immunochemically identical with their unlabeled counterparts. The characterization of labeled factor X_a is described under Results.

Isolation and ^{14}C Labeling of Platelets. Platelets were isolated from bovine blood according to the method of Tollefsen et al. (1974) but with citrate as an anticoagulant instead of EDTA. Nine parts of bovine blood, drawn by jugular vein puncture, were collected in a plastic bottle containing 1 part of 2.85% trisodium citrate solution. The washed platelets were suspended in concentration of approximately 10^8 per mL and 2 μL (0.2 μCi) of [^{14}C]hydroxytryptamine binoxalate (44 μCi per μmol) was added per mL. The suspension was incubated for 30 min at 37 °C, washed twice by centrifugation at 1300*g* for 10 min, and resuspended in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, containing bovine serum albumin (5 mg/mL), glucose (1 mg/mL), and 2.5 mM $CaCl_2$ to a final concentration of 1×10^8 per mL. The platelets were counted in a Technicon platelet autocounter. The isolated platelets had a normal shape when inspected in the phase contrast microscope.

The Release Reaction and Binding Measurements. The release reaction was induced in 2–10 mL of the platelet suspension either by adding 1 unit of bovine thrombin per mL of reaction mixture or the calcium ionophore, A 23 187 (Lilly), to a final concentration of 1 μM. Immediately after the thrombin or Ca ionophore addition, the platelet suspension was carefully mixed. Binding experiments were made at ambient temperature, 22–24 °C, in (11 × 55 mm) polystyrene tubes. The binding of proteins to platelets and platelet release was measured by the oil centrifugation technique (Miletich et al., 1977; Feinberg et al., 1974; Martin et al., 1976). The reaction mixture (0.05–0.1 mL) was carefully layered on 0.1 mL of an oil mixture (1 part apiezon, 9 parts *n*-butyl phthalate) in 0.4-mL plastic centrifuge tubes. After centrifugation in a Beckman microfuge for 1 min, the bottoms of the tubes with the platelet pellets were cut off and the radioactivity was measured in a γ counter. The counting error was less than 2%. Platelet serotonin release was measured by determining [^{14}C]serotonin in aliquots of supernatants from parallel incubations from which iodinated proteins were omitted. Radioactivity was measured in Packard Instagel in a liquid scintillation counter. One hundred percent release was estimated by counting ^{14}C in an aliquot of the platelet suspension without prior centrifugation.

When inspected under the phase contrast microscope, the thrombin and ionophore treated platelets had typical appearance for platelets that have undergone the release reaction. There was no sign of aggregation during the first 30 min but after that time both microscopic and macroscopic aggregation occurred. The aggregation tendency was greatly enhanced when the platelet suspensions were stirred. There was no evidence of platelet lysis using 1 μM Ca ionophore concentration, whereas 2 μM or higher ionophore concentrations induced rapid platelet lysis.

In some dissociation experiments platelet bound factor X_a was separated from free factor X_a by Millipore filtration. The filters were washed with ice-cold buffer, 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, 5 mg/mL bovine serum albumin, 1 mg/mL glucose, and 2.5 mM $CaCl_2$, and radioactivity was measured in a γ counter.

Results

Characterization of Iodinated Factor X_a. Factor X_a had the same electrophoretic mobility on agarose gel electrophoresis prior to and after labeling. Crossed immunoelectrophoresis of labeled factor X_a with monospecific rabbit anti-factor X antiserum and unlabeled factor X_a as carrier showed that factor X_a is less readily recognized by the antiserum than the zymogen. Autoradiography revealed that radioactivity was confined to the immunoprecipitates (Figure 1). For both factor X and X_a, 85% of the radioactivity was recovered in the corresponding protein band on the agarose gel when the immunoprecipitates were cut out and the radioactivity was counted. The cathodal degradation product present in some factor X_a preparations did not bind to platelets. No correction was made for the presence of this protein in the binding studies. [^{125}I]-

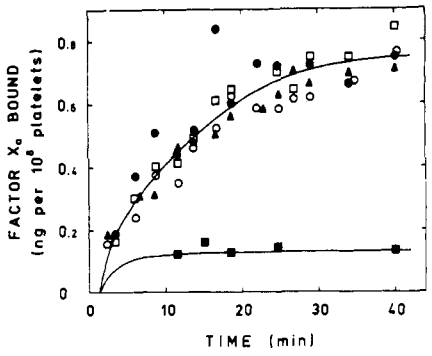

FIGURE 2: Comparison of platelet binding of factor X_a of different specific radioactivities. ^{125}-labeled factor X_a was diluted with unlabeled factor X_a. Parallel platelet suspensions previously treated with 1 unit of thrombin per mL were incubated with 20 ng per mL of the different ^{125}I-labeled factor X_a solutions and the binding to platelets was measured as a function of time. (●) 22 000 cpm per ng of factor X_a (100% labeled factor X_a); (□) 16 500 cpm per ng of factor X_a; (▲) 11 000 cpm per ng of factor X_a; (○) 5500 cpm per ng of factor X_a; (■) nonspecific binding.

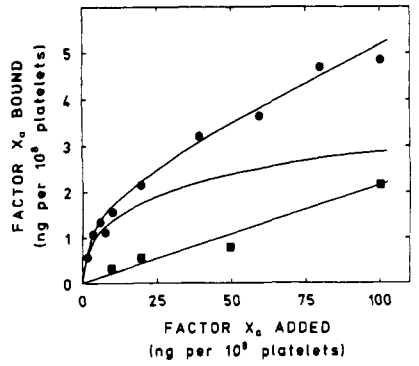

FIGURE 3: Binding of ^{125}I-labeled factor X_a to platelets as a function of the ^{125}I-labeled factor X_a concentration. Before incubation, platelet release was induced with 1 unit of thrombin per mL. Binding was measured after 30-min incubation. Nonspecific binding of ^{125}I-labeled factor X_a was measured in parallel reaction mixtures in the presence of 100-fold excess of unlabeled factor X_a. The specific binding of ^{125}I-labeled factor X_a was obtained by subtraction of the nonspecific binding from the total ^{125}I-labeled factor X_a bound. (●) Binding of factor X_a; (■) nonspecific factor X_a binding; (—) specific factor X_a binding.

Dip-F factor X_a gave the same precipitation pattern as ^{125}I-labeled factor X_a on crossed immunoelectrophoresis.

The rate of thrombin production induced by ^{125}I-labeled factor X_a in the presence of platelets varied between 20 and 100% of that of unlabeled factor X_a. The factor X_a activity of ^{125}I-labeled factor X_a also varied between 20 and 100% of that of unlabeled factor X_a. It was therefore necessary to exclude the possibility of different binding behavior between labeled and unlabeled protein. Factor X_a solutions with different specific activities were made from two labeled factor X_a preparations with low biological activity by mixing iodinated and unlabeled factor X_a. When binding to released platelets was studied as a function of time using these solutions, no significant differences were found (Figure 2). In another experiment when bound factor X_a was measured as a function of total factor X_a concentration, the preparations with different specific activities showed identical binding. More than a fourfold dilution with unlabeled factor X_a was, however, not compatible with precise radioactivity measurements in the platelet pellets. These experiments indicate that despite the slight variation in biological activity of ^{125}I-labeled factor X_a the binding to released platelets of the labeled protein is not differed from that of the unlabeled protein as seen by the identical binding curves.

Binding of ^{125}I-Labeled Factor X_a to Platelets. Binding of factor X_a to platelets, in which the release reaction had been induced with 1 U thrombin per mL, was measured as a function of factor X_a concentration. More than ten experiments were performed with nearly identical results. One experiment is shown in Figure 3. Nonspecific binding was estimated in parallel platelet-^{125}I-labeled factor X_a incubations in the presence of a 100-fold excess of unlabeled factor X_a and used for further corrections. The corrected binding was saturable at levels of 2–3 ng of ^{125}I-factor X_a per 1×10^8 platelets. This corresponds to 290–420 binding sites per platelet according to Scatchard plots. The specific binding required that platelet release had occurred and in most experiments equilibrium was reached after about 15 min, whereas nonspecific binding was rapid and nonsaturable. The amount of factor X_a bound was independent of releasing agent used (thrombin or Ca ionophore). The nonspecific binding to released platelets corresponded to the total binding to unreleased platelets. The degree of binding of factor X_a to the platelets appeared to correlate to the degree of platelet serotonin release as observed in experiments where calcium ionophore concentrations below 1 μM were used. The specific binding required calcium in the incubation mixture. No specific binding of the zymogen, factor X, was observed whether platelet release had been induced or not. The binding data, plotted as bound vs. total factor X_a and a dose-response curve showing the rate of thrombin formation as a function of factor X_a concentration, are shown in Figure 4. The dose-response curve was made with prothrombin concentration of 1.0 mg per mL to ensure that the prothrombin concentration was not rate limiting. The correlation between the factor X_a binding curve and the rate of thrombin formation was good, although the maximal rate of thrombin formation was reached at approximately 50% of the saturating factor X_a concentration. The rate of thrombin formation at each factor X_a concentration was measured from the linear part of the

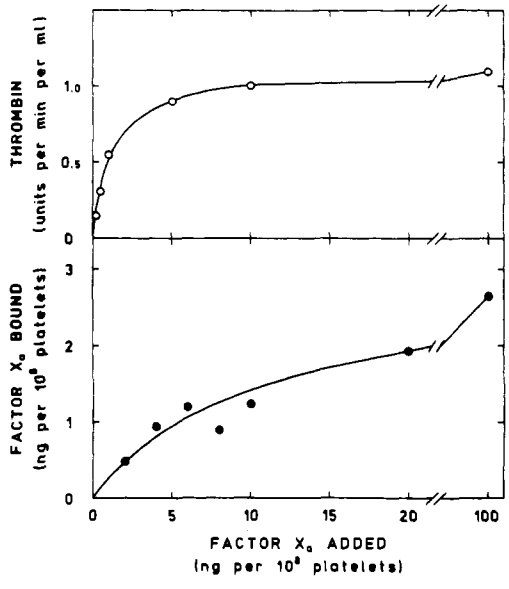

FIGURE 4: Correlation between the rate of thrombin formation and factor X_a binding. Above, rates of thrombin formation in parallel reaction mixtures containing platelets (1×10^8 per mL), prothrombin (1 mg per mL), Ca^{2+} (2.5 mM), and increasing concentrations of factor X_a. Below, binding of ^{125}I-labeled factor X_a to platelets as a function of ^{125}I-labeled factor X_a concentration (no prothrombin present). The platelets were treated with 1 unit of thrombin per mL before the incubation was started. The binding was measured after 30-min incubation and the binding data were corrected for nonspecific binding. (○) Rate of thrombin formation; (●) specific factor X_a binding.

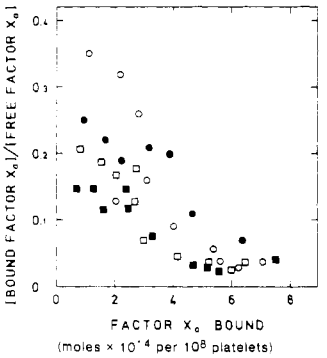

FIGURE 5: Scatchard plots of specific ^{125}I-labeled factor X_a binding data from four different experiments on different days.

TABLE I: Rate and Equilibrium Constants for Binding of Factor X_a to Platelets.

rate constants[a]	
k_{+1}(assoc) (M^{-1} s^{-1})	2.4×10^6 (2.1×10^6 to 2.9×10^6)
k_{-1}(dissoc) (s^{-1})[b]	9×10^{-4} (7×10^{-4} to 12×10^{-4})
assoc constant (M^{-1})[a]	
k_A[c]	5.2×10^9 (2.8×10^9 to 1.0×10^{10})
calcd K_A[d]	2.7×10^9 (1.8×10^9 to 4.1×10^9)

[a] The average from three to six determinations on different days is given with the range in parentheses. [b] Determined by filtration assay (see the text). [c] From Scatchard plots. [d] Calculated from K_{+1}/K_{-1}.

curve obtained after complete platelet release had occurred. Prothrombin was not present in the reaction mixtures used to measure factor X_a binding.

The binding data from four experiments plotted according to Scatchard are shown in Figure 5. The interexperimental variation seen in the figure is largely due to variation inherent in the platelet counting procedure. The binding data are, within experimental error, compatible with the existence of one class of binding sites. The intercept on the abscissa in several experiments indicated a limiting value of 4.8×10^{-14} to 7.0×10^{-14} moles of factor X_a bound per 10^8 platelets (290–420 molecules per platelet) and from the intercept on the y axis an apparent association constant of 2.8×10^9 to 1.0×10^{10} M^{-1} was calculated (Table I). From the dose-response curves the concentrations of factor X_a required to give half-maximal thrombin formation rates were found to be 1–1.5 ng/mL. This would give an estimated association constant of $3–4 \times 10^{10}$ M^{-1}. A possible explanation for this somewhat higher value may have been that there was an influence of prothrombin on the factor X_a binding.

Rate of Binding and Dissociation of Factor X_a–Receptor Complex. Factor X_a binds only to platelets that have undergone the release reaction. Release is generally complete within 2 to 3 min on incubation of 1×10^8 platelets, with 1 U thrombin per mL. When iodinated factor X_a was added with the thrombin, maximum binding was not obtained until after 10 to 20 min. Identical results were obtained with calcium ionophore released platelets. Serotonin is released from dense granules in the platelet whereas the factor X_a receptor might have been associated with some other type of granule. To rule out the possibility that the rate of factor X_a binding depends

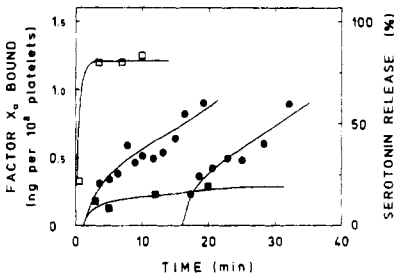

FIGURE 6: Rate of binding of ^{125}I-labeled factor X_a to isolated platelets. One milliliter suspension (10^8 per mL) was incubated with 20 ng of ^{125}I-labeled factor X_a. The incubations were started 75s and 16 min after induction of the release reactions with 1 unit of thrombin. The binding data were not corrected for nonspecific binding. [^{14}C]Serotonin release was measured in a parallel platelet suspension where the labeled factor X_a was omitted. A platelet suspension that had not been treated with thrombin was also incubated with 20 ng per mL of ^{125}I-labeled factor X_a and the binding was followed as a function of time. (●) Factor X_a binding to platelets after the release reaction; (■) binding to platelets that had not undergone the release reaction; (□) serotonin release.

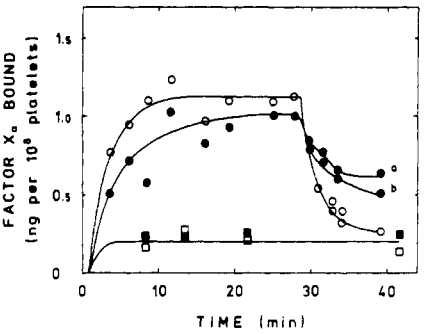

FIGURE 7: Binding of ^{125}I-labeled factor X_a and [^{125}I]Dip-F-factor X_a to platelets as a function of time. The platelet suspension was treated with 1 unit of thrombin per mL, before incubation with 20 ng per mL of ^{125}I-labeled factor X_a or [^{125}I]Dip-F-factor X_a. Binding was measured in aliquots of 50 μL as described in Materials and Methods. After 28 min of incubation, a 100-fold molar excess of unlabeled factor X_a (b) or unlabeled Dip-F-factor X_a (a) was added to portions of the reaction mixtures and the displacement of the labeled proteins measured. (●) Binding of factor X_a; (○) binding of Dip-F-factor X_a; (■) nonspecific binding of factor X_a; (□) nonspecific binding of Dip-F-factor X_a.

on another platelet release reaction that was slower than serotonin release, the rate of factor X_a binding was measured with the centrifugation technique when ^{125}I-labeled factor X_a was added both immediately after the induction of serotonin release and approximately 15 min thereafter. From Figure 6 it is obvious that the rate of binding was identical in both cases.

When the rate of formation of the factor X_a receptor complex was studied, it was noticed that nonspecific binding was always maximal at the first measurement (2–8 min). All binding data were corrected for nonspecific binding as described. Several experiments gave curves identical with those in Figure 7. The average amount of factor X_a bound to 1×10^8 platelets was 6×10^{-14} mol. This number was used as the concentration of receptor for the calculation of the bimolecular rate constant of factor X_a–receptor binding. The rate of association was second order within experimental error with respect to factor X_a concentration. The results are given in Table I.

The rate of dissociation of the factor X_a–receptor complex was measured using both the centrifugation assay after addition of a large excess of unlabeled factor X_a and using the filtration assay with and without addition of an excess of unlabeled factor X_a. In experiments where an excess of unlabeled

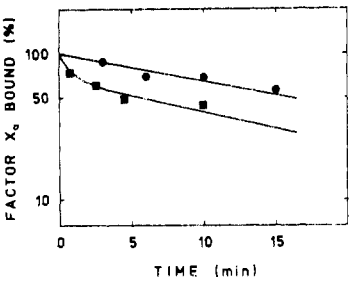

FIGURE 8: Time course of dissociation of specifically bound ^{125}I-labeled factor X_a from platelets (20 ng of labeled factor X_a per 10^8 platelets, equilibrated at 22 °C for 25 min). One set of samples was diluted with 40 volumes of incubation buffer at 22 °C and at the times indicated the samples were filtered under reduced pressure using 1.2-μm Millipore filters and washed with 10 mL of ice-cold incubation buffer. In another set a 100-fold excess of unlabeled factor X_a was added and the displacement of the labeled factor X_a was studied with the oil centrifugation technique. (■) Oil centrifugation experiment; (●) Millipore filtration experiment.

factor X_a was added, the semilog plot was not linear indicating that the process was not first order. When the process was studied using the filtration assay without added unlabeled factor X_a a linear plot was obtained (Figure 8). The results of the oil centrifugation experiments and those Millipore filtration experiments in which a 100-fold excess of unlabeled factor X_a in the diluent was used gave identical results. The results are presented in Table I.

The apparent dissociation constant K_D from several experiments, calculated from $K_D = K_{-1}/K_{+1}$, was 2.4×10^{-10} to 5.7×10^{-10} M using the K_{-1} from the filtration assay where no excess of unlabeled factor X_a was added. This is in good agreement with the K_D value from the equilibrium experiments.

Interaction of Dip-F-Factor X_a with Platelet Receptor. The rate of binding of Dip-F-factor X_a was equal to or faster than that for factor X_a within experimental error when using both thrombin and calcium ionophore released platelets (Figure 7). This makes the requirement for enzymatic, i.e., proteolytic, modification of a receptor protein before actual binding can occur unlikely.

Both ^{125}I-labeled factor X_a and ^{125}I-labeled-factor X_a were effectively displaced from the receptor by a large molar excess of unlabeled Dip-F-factor X_a. From Figure 7 it is obvious that the rate of dissociation of ^{125}I-labeled-factor X_a from platelets by unlabeled factor X_a and Dip-F-factor X_a was more rapid than the dissociation of ^{125}I-labeled factor X_a. Moreover the dissociation of ^{125}I-labeled Dip-F-factor X_a was more complete than that of ^{125}I-labeled factor X_a. This might have been due to the microscopic aggregation of platelets that was observed late in the incubations with factor X_a; i.e., the labeled protein might have been mechanically trapped between aggregating platelets to some extent. Such aggregation was not, however, seen in incubations with ^{125}I-labeled Dip-F-factor X_a. A 100-fold excess unlabeled $PhCH_2SO_2F$-factor X_a also displaced ^{125}I-labeled factor X_a from the receptor.

Displacement of factor X_a from platelets by Dip-F-factor X_a effectively inhibits thrombin formation in the platelet-prothrombin-factor X_a system. In incubations with increasing concentrations of Dip-F-factor X_a, the rate of thrombin formation gradually decreased (Figure 9a). If the Dip-F-factor X_a was added after the reaction had been started by factor X_a, all further prothrombin activation was effectively inhibited as seen in Figure 9b. This indicates that factor X_a was required not only to initiate the reaction but also for the continued prothrombin activation. From Figure 9a it is obvious that it

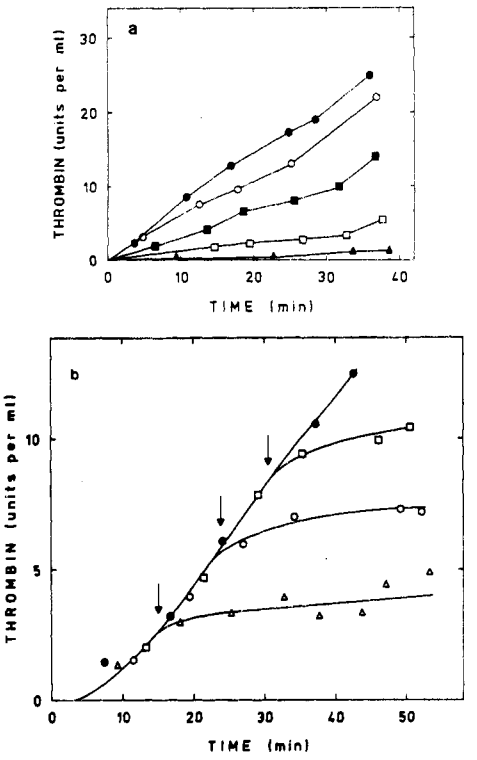

FIGURE 9: Effect of increasing concentrations of Dip-F-factor X_a on rates of prothrombin activation. The prothrombin activation was measured in parallel mixtures of platelets (1×10^8 per mL), prothrombin (0.14 mg per mL), and increasing concentrations of Dip-F-factor X_a. The reaction was started by the addition of factor X_a (11.8 ng per mL). The reaction mixtures contained the following concentrations of Dip-F-factor X_a (○) 24 ng per mL; (■) 59 ng per mL; (□) 118 ng per mL; (▲) 1.6 μg per mL; (●) control without Dip-F-factor X_a. (b) Effect of the addition of an excess of Dip-F-factor X_a on rates of prothrombin activation. The prothrombin activation was measured in parallel mixtures of platelets (1×10^8 per mL), prothrombin (0.14 mg/mL), and factor X_a (11.8 ng per mL). At the times indicated (arrows) a 100-fold excess DIP-factor X_a was added to the different reaction mixtures.

takes about a fivefold molar excess of Dip-F-factor X_a to cause a 50% inhibition of the thrombin formation rate. This is probably due to a somewhat lower association constant for Dip-F-factor X_a binding to the receptor than for factor X_a binding. This is also suggested from Figure 7, which shows that both the rates of binding and of dissociation of Dip-F-factor X_a from platelets is not quite identical with the rates of binding and dissociation of factor X_a.

Comparison of Prothrombin Activation Rates in the Presence of Platelets and in the Presence of Phospholipid and Factor V. The rate of prothrombin activation was measured in the presence of platelets and three different concentrations of factor X_a (Figure 10a). A maximum rate of activation was observed after a short lag phase even at the lowest factor X_a concentration. Doubling the platelet concentration led to a shortening of the lag phase but the maximum rate was not increased. The prothrombin concentration was not rate limiting. The activation rates were compared with those obtained using optimum concentrations of phospholipid and factor V (Figure 10b) instead of platelets. In these experiments activation rates increased with increasing factor X_a concentrations. The maximum rate of activation was similar to that found in the platelet system. Increasing the prothrombin concentration did not lead to higher rates of activation. With factor V and phospholipid there was no lag phase which might have been due to the fact that the specific activity of the factor V preparation was not increased by incubation with thrombin. No

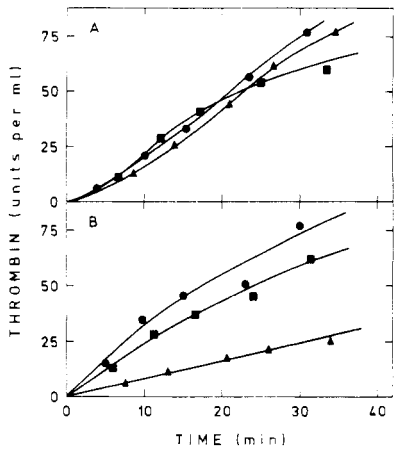

FIGURE 10: Rates of thrombin formation in the presence of platelets and phospholipid and factor V. (A) Prothrombin, 0.14 mg per mL, 2.5 mM $CaCl_2$, and platelets 1×10^8, were incubated with increasing concentration of factor X_a. (B) Same as A except that phospholipid, 0.2 mg per mL, and factor V, 1 unit per mL, were used instead of platelets. The factor X_a concentrations were: (●—●) 34 ng per mL; (■--■) 17 ng per mL; and (▲—▲) 3.4 ng per mL.

measurable thrombin activity formed within 40 min if the factor V or the phospholipid was omitted from the incubation mixture. From these experiments it is obvious that the platelet system is more effective in generating thrombin than is phospholipid–factor V at low factor X_a concentrations. This difference is even larger if the factor X_a concentration is further decreased.

Discussion

The factor X preparations were electrophoretically homogenous before and after activation with RVV and had high clotting activity. A small amount of autolysis product that slowly appeared on storage of the active enzyme did not significantly influence the results. The iodinated factor X_a was native as judged by antibody precipitation tests and bound to platelets in a way that was indistinguishable from that of the unlabeled protein. Furthermore the degree of nonsaturable, "nonspecific" binding to platelets was low and fairly constant from one experiment to another and from one factor X_a preparation to another and most importantly binding correlated with the physiological response, i.e., the rate of thrombin formation. With these criteria fullfilled conclusions drawn on the interaction of native factor X_a with platelets from these experiments should be valid.

The interaction between factor X_a and platelets that had undergone the release reaction by incubation with thrombin or calcium ionophore had the properties of a true receptor interaction and had the same general characteristics as the binding of polypeptide hormones to cell surface receptors (Cuatrecasas & Hollenberg, 1976). The binding was saturable and had high affinity, it was reversible, and the binding data correlated well with the biological dose–response curve. This indicates that a biologically significant interaction was studied. The results obtained in this study confirm and extend the results recently reported by Miletich et al. (1977). Thus the number of binding sites per cell was 290–420 which compares well with the value of 380 human factor X_a molecules bound per platelet at saturation reported by these authors. The rate constant for binding of factor X_a to the platelet receptor was comparable to that obtained for binding of polypeptide hormones to cell surface receptors (Cuatrecasas and Hollenberg, 1976; Cuatrecasas, 1971; Frazier et al., 1974). The rate of dissociation of the factor X_a–receptor complex was substantially increased in the presence of unlabeled factor X_a. Such dissociative behavior has also been found for some polypeptide hormone receptor complexes. Plausible explanations for this may be negatively cooperative binding or receptor heterogeneity (Cuatrecasas & Hollenberg, 1976).

In prothrombin the vitamin K dependent calcium binding structures required for phospholipid binding are all in the aminoterminal part of prothrombin fragment 1. The amino acid sequence in this part of the molecule shows an extensive homology to the amino-terminal γ-carboxyglutamic acid containing part of the light chain of factor X. This would indicate that the phospholipid binding of prothrombin and factor X are similar, although some differences have been reported (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977; Lim et al., 1977). It may thus be noted that the association constant for binding of prothrombin fragment 1 to phospholipid in the presence of calcium ions was found to be in the range of 2×10^5 to 10^6 M^{-1} by Dombrose et al. (1978). This should be compared with the association constant for binding of factor X_a to platelets found in our experiments, i.e., approximately $3\text{–}5 \times 10^9$ M^{-1} from both equilibrium measurements and rate measurements. Miletich et al. (1977) could not demonstrate binding of human factor X to platelets and we could not demonstrate binding of bovine factor X to bovine released platelets nor could we demonstrate binding of prothrombin to the platelets (not published). If the two zymogens have association constants for binding to platelets of the same order of magnitude as Dombrose et al. (1978) found for binding of prothrombin fragment 1 to phospholipid, this is not surprising. Furthermore if one assumes that the rate of formation of zymogen–platelet receptor complex is approximately the same as that for factor X_a binding to platelets the half-life of the complex should be less than a second.

The large difference in association constant between prothrombin fragment 1 and phospholipid and factor X_a and platelets would indicate that platelets catalyze prothrombin activation not only by supplying suitable, i.e., negatively charged, phospholipid but is involved in some other way as well. This is in line with the observation of Marcus et al. (1966) that platelet membrane particles are approximately 20 times more effective than a corresponding amount of platelet membrane lipid in the so called thromboplastin generation test. In contrast to this, however, it was not possible in a more recent study (Vecchione & Zucker, 1975) to demonstrate that platelets provide other procoagulant activity in plasma than phospholipid. In this context it is also noteworthy that we found that at very low factor X_a concentrations the platelet system is more effective than a phospholipid–factor V system, whereas at higher factor X_a concentrations the rates of prothrombin activation are comparable.

In addition to factor X_a, calcium ions, phospholipid, and factor V are part of the prothrombin activation complex. Recently factor V coagulant activity was found in platelets by Breederveld et al. (1975) and by Østerud et al. (1977) who also showed that the factor V activity increased when the platelets were lysed by freezing and thawing. The fact that factor X_a binds to factor V and that factor V can be inactivated by thrombin adds to the similarities of factor V and the platelet factor X_a receptor since Miletich et al. (1977) showed that the receptor was inactivated by high concentrations of thrombin. In this context it should be noted that factor V can bind prothrombin and factor X_a only after it has been activated by thrombin (Suttie & Jackson, 1977; Freeman et al., 1977). Binding of factor X_a to platelets, however, does not require thrombin activation since binding is identical to thrombin and calcium ionophore released platelets as also demonstrated by

Miletich et al. (1977). The possibility that the platelet receptor must be proteolytically modified by factor X_a itself before actual binding can occur seems unlikely since Dip-F-inactivated factor X_a bound like the active enzyme. Whether plasma factor V and the platelet factor X_a receptor are chemically related as are for instance plasma factor XIII and platelet factor XIII, or chemically unrelated is, of course, unclear.

Acknowledgment

One of the authors (J.S.) would like to thank Drs. C. M. Jackson, P. W. Majerus, and J. P. Miletich for demonstrating the oil centrifugation technique during a visit to St. Louis and for making a manuscript (Miletich et al., 1977) available prior to publication.

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