Kinetics of Coagulation Factor X Activation by Platelet-Bound Factor IXa†

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ABSTRACT: Thrombin-activated human platelets, in the presence of factors VIIIa and X, have specific, high-affinity ($K_d \sim 0.5$ nM), saturable binding sites for factor IXa that are involved in factor X activation [Ahmad, S. S., Rawala-Sheikh, R., & Walsh, P. N. (1989) *J. Biol. Chem. 264*, 3244-3251]. To determine the functional consequences of factor IXa binding to platelets, a detailed kinetic analysis of the effects of platelets, phospholipids, and factor VIII on factor IXa catalyzed factor X activation was done. In the absence of platelets, phospholipids, or factor VIII, the Michaelis constant ($K_m = 81$ μM) was >500-fold higher than the factor X concentration in human plasma. Unactivated platelets and thrombin-activated factor VIII, alone or in combination, had no effect on the kinetic parameters, whereas thrombin-activated platelets caused a major decrease in K_m (0.39 μM) with no significant effect on k_{cat} (0.052 min^{-1}) and allowed factor VIIIa to decrease the K_m further to a concentration (0.16 μM) near that of factor X in plasma and to increase the k_{cat} 24000-fold to 1240 min^{-1}. Sonicated mixed phosphatidylserine/phosphatidylcholine vesicles (25/75, mol/mol) had kinetic effects similar to those of activated platelets. When factor IXa binding to thrombin-activated platelets and rates of factor X activation were measured simultaneously at saturating concentrations of factor X and factor VIIIa, the k_{cat} was independent of factor IXa concentration, and the mean k_{cat} value was 2391 min^{-1}. The increase in catalytic efficiency (k_{cat}/K_m) in the presence of thrombin-activated platelets and factor VIIIa was (17.4×10^6)-fold.

Platelets promote interactions between coagulation proteins at various stages of the intrinsic system including contact activation (Walsh & Griffin, 1981), factor IX activation (Walsh et al., 1986), factor X activation (Walsh & Biggs, 1972), and prothrombin activation (Miletich et al., 1977; Tracy et al., 1981). These reactions appear to take place at specific locations on the surface of activated platelets, since platelets have been shown to possess specific, high-affinity saturable receptors for factor XI (Greengard et al., 1986), factor XIa (Sinha et al., 1984), high molecular weight kininogen (Greengard & Griffin, 1984), factor Xa (Miletich et al., 1977; Tracy et al., 1981; Dahlback & Stenflo, 1978), and factors V and Va (Tracy et al., 1979; Kane et al., 1980). In addition, specific, high-affinity, saturable binding sites for both factor IX and factor IXa (Ahmad et al., 1989a), and also for factor VIII (Nesheim et al., 1988), are present on activated human platelets in the presence of calcium ions. Platelets activated by thrombin, collagen, and other agonists can also promote the proteolytic activation of factor X (Walsh & Biggs, 1972; Walsh, 1978; Hultin, 1982; vanRijn et al., 1983; Rosing et al., 1985; Neuenschwander & Jesty, 1988). Previous studies with bovine coagulation proteins indicate that phospholipid vesicles promote factor X activation by factor IXa (Lundblad & Davie, 1964, 1965; Schiffman et al., 1966) primarily by decreasing the Michaelis constant, K_m (vanDieijen et al., 1981), whereas thrombin-activated factor VIII has a major effect on maximal velocity, V_{max} (vanDieijen et al., 1981; Hultin, 1982). The present studies were carried out with human coagulation proteins to examine the kinetic consequences on factor X activation of factor IXa binding to thrombin-activated platelets in the presence and absence of thrombin-activated factor VIII and to compare the kinetic effects of platelets with those of phospholipids.

Experimental Procedures

Materials. The chromogenic substrate S2337 (Bz-Ile-Glu-[γ-piperidyl]Gly-Arg-*p*-nitroanilide) was purchased from AB Kabi Diagnostica (Stockholm, Sweden). Phosphatidylserine from bovine brain was obtained from Serdary Research Laboratories (London, Ontario, Canada) and L-α-dioleoylphosphatidylcholine from Avanti Polar-Lipids, Inc. (Birmingham, AL). D-Phenylalanylprolylarginyl chloromethyl ketone (PPACK)[1] was purchased from Calbiochem-Behring Corp. (San Diego, CA). All other reagents and chemicals used were the same as previously reported (Ahmad et al., 1989a), were obtained from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co., Inc. (Milwaukee, WI), and Calbiochem-Behring Corp. (San Diego, CA), and were of the highest grade commercially available.

Proteins. Human coagulation proteins, including factor IX, factor IXa, factor VIII, factor X, and α-thrombin, were purified, assayed, and characterized as previously published (Ahmad et al., 1989a). All proteins were >98% pure as determined by polyacrylamide slab gel electrophoresis in Na$DodSO_4$ (Laemmli, 1970). Protein concentrations were determined by the Bio-Rad dye binding assay according to instructions provided by the manufacturer (Bio-Rad, Richmond, CA).

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[1] Abbreviations: PPACK, D-phenylalanylprolylarginyl chloromethyl ketone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; Na$DodSO_4$, sodium dodecyl sulfate.

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Table I: Factor IXa Catalyzed Factor X Activation: Kinetic Analysis

additions	kinetic parameters				
	K_m (μM)	V_{max} (nM·min^{-1})	k_{cat}[a] (min^{-1})	k_{cat}/K_m (μM^{-1}·min^{-1})	x-fold increase in catalytic efficiency
FIXa	81	0.35	0.07	0.86×10^{-3}	1
FIXa, FVIIIa (k units/mL)	34	0.29	0.058	1.7×10^{-3}	2.0
FIXa, unactivated platelets (5×10^7/mL)	45	0.46	0.092	2.0×10^{-3}	2.3
FIXa, unactivated platelets (5×10^7/mL), FVIIIa (5 units/mL)	45	0.35	0.07	1.6×10^{-3}	1.9
FIXa, PS/PC (5 μM)	0.14	0.48	0.048	0.34	395
FIXa, PS/PC (10 μM)	0.20	0.57	0.057	0.29	337
FIXa, PS/PC (25 μM)	0.94	0.95	0.095	0.10	116
FIXa, PS/PC (200 μM)	10.11	7.92	0.79	0.078	91
FIXa, thrombin-treated platelets (5×10^7/mL)	0.39	0.52	0.052	0.13	151
FIXa, FVIIIa (5 units/mL), PS/PC (25 μM)	0.19	17.4	1740	9158	10.6×10^6
FIXa, FVIIIa (5 units/mL), thrombin-treated platelets (5×10^7/mL)	0.16	12.4	1240 (2391)[b]	7750 (14944)	9.0×10^6 (17.4×10^6)

[a] k_{cat} expressed as moles of FXa formed per mole of total added FIXa. [b] k_{cat} expressed as moles of FXa formed per minute per mole of total platelet-bound FIXa.

Phospholipids and Phospholipid Vesicle Preparation. Phospholipid vesicles were prepared in an atmosphere of nitrogen from a mixture of phosphatidylserine and L-α-dioleoylphosphatidylcholine (25/75, mol/mol) and sonicated for 30 s on ice in a buffer containing 50 mM Tris-HCl and 175 mM NaCl (pH 7.9) as previously described (vanDieijen et al., 1981).

Measurement of Rates of Factor Xa Formation. Activation of factor X by factor IXa at 37 °C either in the presence or in the absence of phospholipids, platelets, and/or factor VIIIa (for further experimental conditions and concentrations of reactants, see legend to Figure 1) was monitored by incubating purified factor X, factor IXa, and 5 mM $CaCl_2$ at 37 °C in a buffer containing 50 mM Tris-HCl, 175 mM NaCl, and 0.5 mg/mL human serum albumin (pH 7.4). In separate experiments, the linearity of initial rates of factor X activation was confirmed under all experimental conditions. In experiments with platelets, gel-filtered platelets, prepared as previously described (Ahmad et al., 1989a), were incubated for 10 min at 37 °C with human α-thrombin (0.1 unit/mL) in the presence of factor IXa and $CaCl_2$ prior to the addition of PPACK (50 nM) to neutralize the thrombin. Similar results were obtained when PPACK was not added (see Results and Discussion), and PPACK (50 nM) had no effect on factor IXa or factor Xa (data not shown). In some experiments, factor VIII was first preincubated at a concentration of 300 units/mL with human α-thrombin (0.01 unit/mL) for 1 min at 37 °C before addition at a concentration of 5 units/mL to the incubation mixture to which factor X was added to initiate factor X activation. After appropriate incubation times at 37 °C, 6 mM EDTA was added to prevent further activation of factor X by factor IXa. Two minutes later, the factor Xa specific chromogenic substrate S2337 (0.6 mM) was added to the reaction mixture to bring the final volume to 350 μL. Factor IXa alone had no amidolytic activity against S2337. After a further 10-min incubation at 37 °C (during which period the rates of cleavage of S2337 were linear), the reaction was stopped by the addition of glacial acetic acid (12.5% final concentration), and the amount of *p*-nitroanilide released was measured spectrophotometrically at 405 nm in microtiter plates in a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). From a linear calibration curve made with known amounts of factor Xa (factor X activated with Russel's viper venom) under the same conditions as described above, the amount of factor Xa present in the aliquot was calculated. The rate of factor Xa formation in the reaction mixture was calculated from the amounts of factor Xa present in the samples. The amount of factor IXa present in the incubation mixture was chosen such that 2–5% or less of added factor X was converted to factor Xa during the time course of the experiment.

Calculation of Kinetic Constants. The derivation of kinetic constants for factor X activation by factor IXa was based on a one-enzyme, one-substrate model. Values reported for the Michaelis constant (K_m) and the maximum velocity (V_{max}) were calculated from the mean ± standard error of the mean (SEM) of four to eight independent determinations each done in duplicate of factor X activation rates at variable factor X concentrations by using a nonlinear regression data analysis program designated Enzfitter (Leatherbarrow, 1985), obtained from Elsevier Science Publishers BV (Amsterdam, The Netherlands), and an IBM Personal System/2 Model 30 computer. To determine turnover numbers (k_{cat}) for platelet-bound enzyme, simultaneous measurements of factor X activation and factor IXa binding were made. The binding of ^{125}I-labeled factor IXa to thrombin-activated, gel-filtered platelets was measured by centrifugation of platelets and bound ligand through silicone oil barriers as previously described (Ahmad et al., 1989a).

Results and Discussion

Our previous studies (Ahmad et al., 1989a) demonstrate the presence of specific, saturable, high-affinity binding sites for factor IXa (~550 per platelet) on thrombin-activated platelets, the affinity of which increased 5-fold (from a K_d of ~2.5 nM to ~0.5 nM) in the presence of saturating concentrations of both thrombin-activated factor VIII and factor X. Factor IXa binding was shown to require the presence of Ca^{2+} ions and the activation of platelets with thrombin. Although factor IX was shown to share ~250 sites per platelet with factor IXa in the absence of factor VIIIa and factor X, the K_d for factor IX binding was ~2.5 nM both in the presence and in the absence of factors VIIIa and X, and factor IXa binding to its high-affinity site (K_d ~0.5 nM) was saturable in the presence of a 400-fold molar excess of factor IX, so that factor IXa can bind to its platelet receptor in the presence of a concentration of factor IX (440 nM) well in excess of that present in normal human plasma (70–100 nM; Thompson, 1977).

To examine the mechanism by which the specific factor IXa receptor functions, we determined the kinetic parameters for the factor IXa catalyzed activation of factor X in the absence and presence of platelets and factor VIII (Figure 1 and Table I). To determine K_m and V_{max} under each experimental

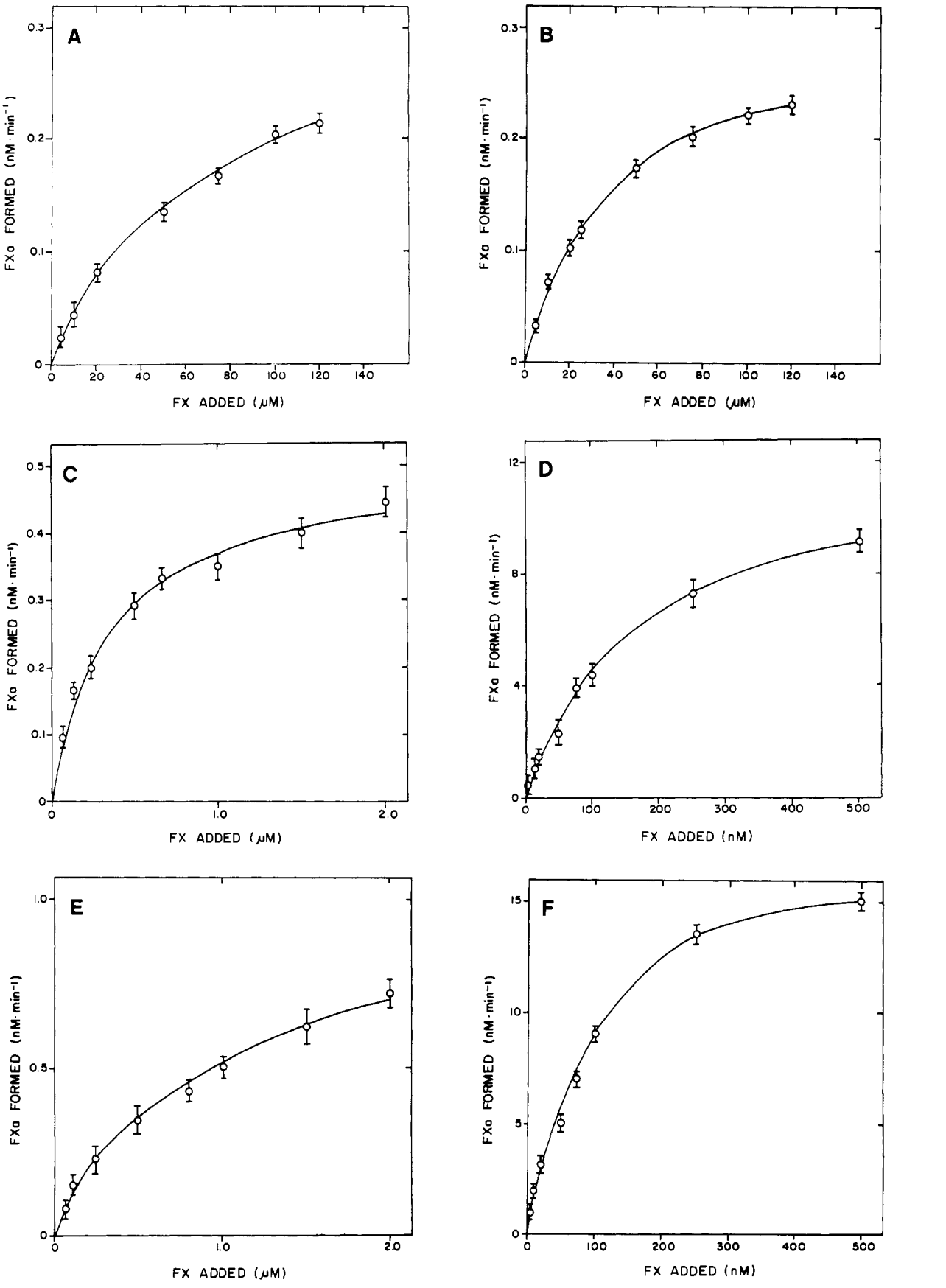

FIGURE 1: Factor X activation by factor IXa. Initial rates of factor X activation (mean ± SEM) were determined as described under Experimental Procedures at various concentrations of factor X as indicated in the graphs. Results plotted in panel A ($n = 5$) are those obtained with factor IXa (5 nM) alone; panel B ($n = 6$), factor IXa (5 nM) and factor VIIIa (5 units/mL); panel C ($n = 5$), thrombin-activated platelets [(3–4) × 10^7/mL] and factor IXa (5 nM); panel D ($n = 6$), thrombin-activated platelets [(3–4) × 10^7/mL], factor IXa (10 pM), and factor VIIIa (5 units/mL); panel E ($n = 4$), phospholipid vesicles (25 μM) and factor IXa (5 nM); panel F ($n = 8$), phospholipid vesicles (25 μM), factor IXa (10 pM), and factor VIIIa (5 units/mL).

condition, concentrations of reactants were established such that substrate (factor X) concentration could be varied at a given factor IXa concentration to give linear rates of factor Xa formation with conversion of <5% (usually <2%) of substrate (factor X) to product (factor Xa). In each case, we obtained saturable kinetics yielding hyperbolic plots (Figure 1) of factor X activation rates versus added factor X concentrations, and linear double-reciprocal plots (Segel, 1975). The kinetic parameters we derived for factor IXa catalyzed factor X activation in the presence of Ca^{2+} ions without added factor VIII, phospholipids, or platelets included a K_m of 81 μM and a k_{cat} of 0.07 mol of factor Xa formed per minute per mole of factor IXa added (Table I). These values are comparable with those obtained by van Dieijen et al. (1981) for bovine factor IXa and factor X, i.e., K_m = 181 μM, k_{cat} = 0.0105 min^{-1}. The K_m value is >500-fold higher than the factor X concentration (0.15 μM) in normal human plasma, indicating that in the absence of accessory factors, the reaction would be highly unfavorable kinetically.

When thrombin-activated factor VIII, unactivated platelets, or unactivated platelets plus factor VIIIa were added to the incubation mixture and the kinetics of factor IXa catalyzed factor X activation determined, very little, if any, effect on either K_m or V_{max} was observed (Table I). This result is particularly important since equilibrium binding studies carried out in parallel (data not shown) and those previously reported (Ahmad et al., 1989a) showed no significant binding of factor IXa to unactivated platelets, and factor VIII has been shown to bind to activated but not to unactivated platelets (Nesheim et al., 1988). In contrast, when thrombin-activated platelets (5×10^7/mL) were present in the reaction mixture, the apparent K_m was decreased from 81 to 0.39 μM, whereas the k_{cat} was unchanged at 0.052 min^{-1}. The further addition of a saturating amount of thrombin-activated factor VIII (5 units/mL) further decreased the K_m to a concentration (0.16 μM) almost identical with that of factor X in human plasma and caused a 24000-fold increase in k_{cat} to 1240 min^{-1}. These experiments were carried out both in the presence and in the absence of PPACK (50 nM) which was added to neutralize the thrombin (0.1 unit/mL) used to activate the platelets so that the added factor VIII (previously activated with 0.01 unit/mL thrombin) would not be inactivated by the higher thrombin concentrations used to activate the platelets. The presence of PPACK (50 nM) did not inactivate the factor IXa added or the factor Xa generated, in agreement with the findings of Jesty (1986) and of Neuenschwander and Jesty (1988). Furthermore, values of K_m and V_{max} were not significantly different in the absence and presence of PPACK (50 nM) in experiments with platelets or phospholipids in the absence of factor VIII (data not shown). In the presence of thrombin-activated factor VIII, the values of k_{cat} were 2.5–3-fold higher in the presence of PPACK (50 nM) than in its absence whereas K_m values were unaffected, suggesting that the presence of PPACK protected factor VIIIa from inactivation by thrombin, but had no effect on factor IXa added or factor Xa generated.

The k_{cat} values determined in the presence of platelets are not strictly valid since a true turnover number should reflect the number of moles of product formed per minute per mole of *active* enzyme (Segel, 1975). Since the K_m for solution-phase activation of factor X by factor IXa (81 μM) is 40–150-fold higher than the maximum concentrations used to determine the K_m in the presence of platelets (Figure 1C,D), it is clear that the rates of factor X activation do not reflect a significant contribution from soluble proteins and thus must arise from platelet-bound factor IX. Therefore, it was important to measure bound factor IXa in the same experiment with determination of rates of factor X activation. When this was done at six different concentrations of added factor IXa, and the k_{cat} expressed as moles of factor Xa formed per minute per mole of bound (i.e., functionally active) factor IXa, the k_{cat} was found to be independent of enzyme concentration (as it should be), and the mean (±SEM) of six k_{cat} values was 2391 (±90) min^{-1}. These data are presented in detail elsewhere (Ahmad et al., 1989b) and are summarized in Table I for comparison with the present detailed kinetic analysis. Therefore, compared with values obtained in the absence of platelets and factor VIIIa, the fold increase in k_{cat} was >34000, whereas the fold increase in catalytic efficiency (k_{cat}/K_m) was 17.4×10^6.

To compare the effects of platelets with phospholipids and to provide a basis for comparison of our work with published studies on bovine coagulation proteins (Lundblad & Davie, 1964, 1965; Schiffman et al., 1966; vanDieijen et al., 1981, 1985), we examined the effects on factor X activation of mixed, sonicated phosphatidylserine/phosphatidylcholine vesicles at various concentrations in the absence and presence of factor VIIIa (Figure 1 and Table I). The kinetic effects of phospholipids were similar to those of thrombin-activated platelets, namely, a major decrease in K_m without an effect on k_{cat} unless factor VIIIa was also present. The results obtained with human proteins are very similar to those reported by van-Dieijen et al. (1981) using bovine proteins. However, although Hultin (1982) reported a K_m value for the activation of tritiated bovine factor X by human factor IXa in the presence of phospholipids and factor VIIIa of 0.14 μM (in close agreement with our K_m of 0.16 μM), the k_{cat} reported by Hultin (1982) was only 24 min^{-1}, compared with our value of 2391 min^{-1}. The reason for this discrepancy is unknown but may be a reflection of different methods used to measure rates of factor X activation, i.e., chromogenic assay in the present work and measurement of a tritium-labeled activation peptide from factor X by Hultin (1982).

When the concentration of phospholipid vesicles was varied from 5 to 200 μM, and the kinetics of factor X activation were examined in the absence of factor VIIIa, a progressive 70-fold increase in the apparent K_m was observed with an associated progressive 17-fold increase in V_{max} and a resultant decrease in catalytic efficiency (Table I). Very similar results have been reported in studies with bovine proteins of the factor IXa catalyzed activation of factor X (vanDieijen et al., 1981) and of factor X activation of factor VIIa tissue factor (Zur & Nemerson, 1980) when the concentration of mixed phospholipids was varied. The mechanism of these effects is not defined, but it has been suggested that the lipid effectively competes with the enzyme "by complexing the substrate in a form that limits accessibility to the enzyme" (Zur & Nemerson, 1980).

All our present studies were carried out with thrombin-activated factor VIII, since Neuenschwander and Jesty (1988) have shown that linear rates of factor X activation require the presence of fully activated factor VIII and that thrombin-activated factor VIII is about twice as active in factor X activation as factor VIII activated by factor Xa. Neuenschwander and Jesty (1988) have also examined the effects of platelets (activated with ionophore A23187) or phospholipids on factor X activation in the presence of optimally activated factor VIII as a function of factor IXa concentration and have derived an apparent dissociation constant of 74 pM "for the factor IXa–VIIIa interaction" in the presence of ionophore-

activated platelets. By comparison, we have determined an apparent K_d of 0.5 nM for factor IXa binding to thrombin-activated platelets in the presence of factor VIIIa and factor X both from equilibrium binding studies (Ahmad et al., 1989a) and from kinetic studies of factor X activation (Ahmad et al., 1989b).

The present results and additional studies reported elsewhere (Ahmad et al., 1989b) indicate a close correspondence between factor IXa binding to platelets and rates of factor X activation and give rise to the hypothesis that platelet receptor occupancy with factor IXa (Ahmad et al., 1989a,b) and factor VIII (Nesheim et al., 1988) or factor VIIIa is essential for the assembly of the factor X activating complex on the activated platelet surface. Although it has been demonstrated that factor VIII binds specifically and reversibly to high-affinity (3.0 nM) sites on activated platelets, and the presence of platelets may enhance the activation of factor VIII to factor VIIIa (Hultin, 1982; Neuenschwander & Jesty 1988), it is not known whether factor VIIIa can bind to platelets or whether factor VIII bound to platelets is converted to factor VIIIa. The similar kinetic effects of phospholipid vesicles and those of platelets are consistent with the hypothesis (vanRijn et al., 1983; Rosing et al., 1985) that exposure of aminophospholipids on the external plasma membrane of platelets is essential for the platelet contribution to factor X activation. However, whether factor IXa binding to its platelet receptor is sufficient to cause these dramatic kinetic effects, or whether the exposure of aminophospholipids is also necessary for factor IXa binding or for factor X activation, is not addressed by the present studies.

These studies with human proteins of the effects of platelets and factor VIIIa on factor IXa catalyzed factor X activation can be compared with studies with bovine proteins of the effects of tissue factor and phospholipids on factor VIIa catalyzed factor X activation. The K_m determined here in the presence of activated platelets and factor VIIIa is 0.16 μM (i.e., almost identical with the concentration of factor X in plasma) which is close to the K_m of 0.34 μM determined by Silverberg et al. (1977) for the factor VIIa tissue factor catalyzed reaction. Similarly, the k_{cat} derived from the present studies for platelet-bound factor IXa in the presence of factor VIIIa is 2391 min^{-1} compared with 1920 min^{-1} for factor VIIa and tissue factor (Silverberg et al., 1977).

The kinetic effects of platelets or phospholipids and factor VIII on factor X activation, combined with evidence of specific interactions of factor IXa (Ahmad et al., 1989a,b) and factor VIII (Nesheim et al., 1988) with activated platelets, suggest some similarities between the mechanism of factor X activation and that of prothrombin activation, as well as some differences. The presence of phospholipids or platelets together with factor Va has been shown to result in a 300 000-fold increase in the rate of prothrombin activation (Jobin & Esnouf, 1967; Esmon et al., 1974; Nesheim et al., 1979). The kinetic mechanism of this rate enhancement has been studied with phospholipid vesicles, which decrease the K_m for bovine prothrombin activation from well above to below the concentration in bovine plasma of prothrombin and permit factor Va to increase the k_{cat} (Rosing et al., 1980), in similar fashion to the kinetic effects of platelets, phospholipids, and factor VIIIa in factor X activation shown in the present paper. Although it has been shown that specific binding of both factor Xa and factor Va to platelets is required for optimal rates of prothrombin activation (Miletich et al., 1977; Dahlback & Stenflo, 1978; Tracy et al., 1979), the interaction of factor Xa with platelets apparently requires either prior or coordinate receptor occupancy by factor Va. In contrast, our studies indicate that binding sites for factor IXa are present on activated platelets in the absence of added factor VIII, the presence of which (together with factor X) enhances the binding affinity 5-fold (Ahmad et al., 1989a,b). Additional differences in the mechanisms of factor X activation and prothrombin activation include the following: (1) Factor Xa can apparently bind to unactivated platelets provided factor Va is expressed on the platelet surface (Miletich et al., 1977, 1978; Dahlback & Stenflo, 1978; Tracy et al., 1979, 1981), whereas the binding of both factor IXa (Ahmad et al., 1989a,b) and factor VIII (Nesheim et al., 1988) to platelets as well as the contribution of platelets to factor X activation (present studies; Walsh & Biggs, 1972; Walsh, 1978; Rosing et al., 1985; Neuenschwander & Jesty, 1988) requires platelet activation. (2) Whereas factor X does not compete with factor Xa for platelet binding sites (Miletich et al., 1977, 1978; Dahlback & Stenflo, 1978), and there is no published evidence of a specific interaction of factor X with platelets, our studies indicate that factor IX shares ~250 low-affinity sites (K_d ~2.5 nM) per platelet with factor Xa (Ahmad et al., 1989a) and that factor IX binding to activated platelets may function to promote factor IX activation by platelet-bound factor XIa (Walsh et al., 1986).

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Examination of the Substrate Specificity of Heparin and Heparan Sulfate Lyases†

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ABSTRACT: We have examined the activities of different preparations of heparin and heparan sulfate lyases from *Flavobacterium heparinum*. The enzymes were incubated with oligosaccharides of known size and sequence and with complex polysaccharide substrates, and the resulting degradation products were analyzed by strong-anion-exchange high-performance liquid chromatography and by oligosaccharide mapping using gradient polyacrylamide gel electrophoresis. Heparinase (EC 4.2.2.7) purified in our laboratory and a so-called Heparinase I (Hep I) from a commercial source yielded similar oligosaccharide maps with heparin substrates and displayed specificity for di- or trisulfated disaccharides of the structure →4)-α-D-GlcNp2S(6R)(1→-4)-α-L-IdoAp2S(1→ (where R = *O*-sulfo or OH). Oligosaccharide mapping with two different commercial preparations of heparan sulfate lyase [heparitinase (EC 4.2.2.8)] indicated close similarities in their depolymerization of heparan sulfate. Furthermore, these enzymes only degraded defined oligosaccharides at hexosaminidic linkages with glucuronic acid: →4)-α-D-GlcNpR(1→4)-β-D-GlcAp(1→ (where R = *N*-acetamido or *N*-sulfo). The enzymes showed activity against solitary glucuronate-containing disaccharides in otherwise highly sulfated domains including the saccharide sequence that contains the antithrombin binding region in heparin. A different commercial enzyme, Heparinase II (Hep II), displayed a broad spectrum of activity against polysaccharide and oligosaccharide substrates, but mapping data indicated that it was a separate enzyme rather than a mixture of heparinase and heparitinase/Hep III. When used in conjunction with the described separation procedures, these enzymes are powerful reagents for the structural/sequence analysis of heparin and heparan sulfate.

Heparin and heparan sulfate are complex, sulfated copolymers of alternating 1→4-linked glucosamine and hexuronic acid. Both biopolymers have been implicated in a diverse range of biological functions and therapeutic uses [for reviews, see Gallagher et al. (1986) and Linhardt and Loganathan (1989)].

Polysaccharide lyases (EC 4.2.2) are a class of enzymes that depolymerize certain acidic polysaccharides through an eliminative mechanism. This enzymatic reaction results in an unsaturated uronic acid residue at the nonreducing terminal sugar in the resulting oligosaccharide product (Linhardt et al., 1986a). *Flavobacterium heparinum* is a soil isolate (Payza & Korn, 1956) capable of utilizing either heparin or heparan sulfate as its sole carbon and nitrogen source (Galliher et al., 1981). The growth of this organism on these polymeric substrates is dependent on its production of a variety of enzymes including lyases, glucuronidases, sulfoesterases, and sulfamidases (Galliher et al., 1982). These polysaccharide lyases

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