We have recently shown that thrombin-stimulated human platelets have specific, saturable receptors for factor IXa, occupancy of which promotes factor X activation (Ahmad, S. S., Rawala-Sheikh, R., and Walsh, P. N. (1989) J. Biol. Chem. 264: 3244–3251, 20012–20016; Rawala-Sheikh, R., Ahmad, S. S., and Walsh, P. N. (1990) Biochemistry 29: 2606–2611). To study the structural requirements for factor IXa binding to platelets, equilibrium binding studies and kinetic studies of factor X activation were carried out with normal factor IXa and with two variant proteins: factor IXaAlabama (FIXaAL: Asp77 → Gly substitution) and factor IXaChapel Hill (FIXaCH: Arg145 → His substitution). In the absence of factors VIIIa and X, there were 331 binding sites/platelet for FIXaCH (Kd_app = 2.8 nM), and 540 sites/platelet for FIXaAL (Kd_app = 3.2 nM), compared with 540 sites/platelet for FIXaCH (Kd_app = 2.3 nM) for normal factor IXa. The addition of factors VIIIa and X, both at saturating concentrations, had no effect on the number of binding sites for either normal or variant factor IXa, resulted in a decrease in the Kd for normal factor IXa to 0.67 nM, resulted in a suboptimal decrease in Kd for FIXaAL (1.4 nM), and had no effect on the Kd for FIXaCH.

Kinetic studies of factor X activation at variable factor IXa concentration confirmed these values of Kd in the presence of factors VIIIa and X. Determination of rates of factor X activation at variable substrate concentrations yielded normal values of catalytic efficiency (kcat/Km) for the variant proteins, thereby indicating that the abnormally low rates of factor X activation obtained were a consequence of the low affinity binding of FIXaAL and FIXaCH to thrombin-activated platelets in the presence of factors VIIIa and X. These studies suggest that the presence of Asp77 and the cleavage of factor IX at Arg145 Ala146 are important structural features required for specific, high affinity factor IXa binding to platelets in the presence of factors VIIIa and X.

Activated human platelets promote the activation of factor X by factor IXa (1, 2). Previous studies from our laboratory, aimed at elucidating the mechanisms by which platelets and factor VIII contribute to this coagulation reaction, have shown that thrombin-stimulated human platelets have specific, saturable binding sites for factor IXa and that the presence of factor VIII and factor X increases the binding affinity 5-fold (3). We have also shown that platelet receptor occupancy with factor IXa is closely correlated with rates of factor X activation (4, 5).

To study the structural requirements for factor IXa binding to platelets, we have now carried out detailed comparative platelet binding and kinetic studies with normal and variant factor IXa molecules. One of these proteins, factor IXaAlabama (factor IXaAL),1 can be activated by factor Xa in the presence of calcium ions to a factor IXa form with about 10% of the clotting activity of the normal factor IXa (6, 7). An adenine to guanine transition in the first nucleotide of exon d causes the substitution of a glycine codon (GGT) for the normal aspartic acid codon (GAT). This point mutation results in a single amino acid substitution at residue 47 of the zymogen in the first epidermal growth factor-like domain of factor IXaAL (8). The factor IXa defects previously reported in the first epidermal growth factor-like domain are mostly associated with mild hemophilia B. In addition to factor IXaAL, these include factor IXaLondon (Pro55 → Ala, 10% of normal activity) (9), factor IXaDarwin (Gly6 → Ser, 14% of normal activity) (10), factor IXaLondon (β-OH Asp44 → Gly, 8% of normal activity), and factor IXaNewLondon (Gluα → Pro, <1% of normal activity) (11).

The second variant protein we have studied is factor IXaChapel Hill (factor IXaCH). The molecular defect in factor IXaCH is the substitution of histidine for arginine at position 145 (12–14). This is the first cleavage site in the normal pathway of factor IX activation. Thus, factor IXaCH is not activated normally either by factor Xla or by factor VIIIa-tissue factor. Only the Arg196–Val181 bond is cleaved giving rise to factor IXaα, in which the activation peptide remains covalently attached to the light chain. Factor IXααCH has 20% of the clotting activity of normal factor IXa. Mutations in certain abnormal factor IX proteins have been demonstrated to cause abnormally slow or incomplete activation of factor IX by factor Xa. These include defects such as that in factor IXaNew, in which cleavage of the Arg196–Val181 bond is prevented by substitution of glutamine for arginine at position 180. The dysfunction of factor IXaNewLondon has also been attributed to

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1 The abbreviations used are: factor IX, factor IXaAlabama, factor IXaChapel Hill, factor IXaLondon, normal factor IX; PFACK, diphenylalanyl-prolyl-arginy1 chloromethyl ketone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
defective cleavage at the Arg^{180}-Val^{181} bond (11).

The lipid binding and kinetic properties of normal factor IXa (factor IXaN) have been examined and compared in detail with the two variant proteins, namely factor IXa AI and factor IXa CH (15). In this paper we have carried out detailed studies to compare the platelet binding and kinetic studies of these abnormal variant factor IXa molecules with factor IXa N.

**EXPERIMENTAL PROCEDURES**

**Materials**—Details of the purification, assay, and characterization of human coagulation proteins, including factor IX, factor IXa, factor VIII, factor X, and α-thrombin, were previously published (3). The conditions used for activation of factor VIII were identical with those previously published (3-5). The variant factor IX molecules were isolated from human plasma as described previously (15). Both the normal and variant factor IX molecules were radiolabeled with 125I by the Iodo-Gen method as previously described (3), and specific radioactivities of all proteins were in the range of 2.0-2.5 × 10^6 cpm/μg. Activation of purified factor IXN, factor IXa AI, and factor IXa CH by purified human factor Xa were carried out as previously described (3). Autoradiograms of normal and variant factor IX and factor IXa were developed to provide structural characterization of 125I-labeled proteins. Both 125I-labeled factor IXa AI and factor IXa CH appeared as single bands at M, = 57,000 (Fig. 1, lanes 2 and 3) and were indistinguishable from factor IXN (Fig. 1, lane 1). Similar results were obtained with thezymogens after reduction (Fig. 1, lanes 4, 5, and 6). Both factor IXa AI (Fig. 1, lane 7) and factor IXa CH (lanes 8) migrated nonreduced at an apparent M, of ~45,000, whereas factor IXa CH (lane 9) remained at a M, of 57,000 since the activation peptide remained covalently attached to the light chain. After reduction, the 125I-labeled factor IXa AI, migrated as two polypeptides of M, = 27,000 and 17,000 (Fig. 1, lane 11), as did factor IXa CH (Fig. 1, lane 10), whereas 125I-labeled factor IXa CH appeared under reducing conditions (Fig. 1, lane 12) as two bands, in addition to a small quantity (8.9% by scanning of the autoradiogram), as previously reported (13) of the original zymogen. One of the two major bands migrated with a mobility identical with that of the heavy chain band from factor IXa CH, whereas the other band had an apparent M, of 45,000 which corresponds to a cleavage product consisting of the light chain and the activation peptide similar to that found on cleavage of either factor IX or factor IXa by Russell’s viper venom (13, 16). The clottings of activated factor IXa AI and factor IXa CH were 15% and 18%, respectively, of the clotting activity of factor IXa CH. Protein concentrations were determined by the Bio-Rad dye binding assay according to instructions provided by the manufacturer. Polyacrylamide slab gels were reduced in NaDodSO₄ and run on a slab gel electrophoresis in NaDodSO₄, was carried out according to the procedure of Laemmli (17).

**Binding Experiments**—In a typical binding experiment, gel-filtered platelets (3-4 × 10⁹/ml) in calcium-free HEPES Tyrode’s buffer, pH 7.4, were incubated at 37 °C in a 1.5-ml Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled factor IXa, calcium chloride, platelet stimulii, and other proteins. Platelets were separated from unbound proteins as previously described (3). The data were analyzed and the number of binding sites and dissociation constants (Kd) were calculated from the means of six independent determinations, each done in duplicate, as previously described (3) using a Mac Plus Computer and the LIGAND Program as modified by McPherson (22).

**Measurements of Rates of Factor Xa Formation**—The activation of factor X by normal and variant factor IXa was carried out at 37 °C in the presence of thrombin-stimulated gel-filtered platelets, factor VIIIa, and calcium chloride as previously described (3-5). The details of experimental conditions and concentrations of reactants are given under “Results” and in the figure legends.

**Calculations of Kinetic Constants**—The derivation of kinetic constants of factor X activation by factor IXa was based on a one-enzyme, one-substrate model. The Michaelis constant (Km) and the maximum velocity (Vmax) were calculated from the mean ± S.E. of five independent determinations each done in duplicate of factor X activation rates at variable factor X concentrations as described previously (5). Values of dissociation constant (Kd) were obtained from experiments in which rates of factor X activation were determined at variable factor IXa concentrations as previously described (4). The values of turnover numbers (kcat) were calculated by dividing Vmax values either by the total factor IXa concentration or by the amount of enzyme (factor IXa) bound under the conditions of the experiment. This latter value was obtained from the equation:

\[ \text{Amount bound} = \frac{B_{\text{max}} \cdot E}{K_d + E} \]

where \( B_{\text{max}} \) is the maximum amount of factor IXa bound or the total receptor concentration; \( E \), total factor IXa concentration; and \( K_d \), dissociation constant. The details of this calculation are provided in previous papers (4, 18).

**RESULTS**

**Specific Binding of 125I-Labeled Factor IXaN, Factor IXaAI, and Factor IXaCH to Thrombin-activated Normal Human Platelets**—In the present work, we have compared binding of 125I-labeled factor IXa with the binding of factor IXa AI and factor IXa CH to normal human platelets. Scatchard analysis of the binding data (Fig. 2) gave straight lines indicating the presence of a single class of binding sites for both the normal and variant factor IXa molecules both in the presence and absence of factor VIIIa and factor X. The affinity and stoichiometry of binding for these ligands under both experimental conditions was determined in six separate experiments, the means (±S.E.) of which are given in Table I. In addition, the stoichiometry and affinity of factor IX binding was determined as previously reported (3), and the results were recorded in Table I. In the absence of factor VIIIa and factor X, there were 331 binding sites/platelet for factor IXa AI (Kd app = 2.8 nM) and 540 sites/platelet for factor IXa AI (Kd app = 3.2 nM), compared with 540 sites/platelet (Kd app = 2.5 nM) for factor IXa CH, and 306 sites/platelet (Kd app = 2.6 nM) for factor IXN. The addition of factor VIIIa and factor X, both at saturating concentrations, had no effect on the number of binding sites for either normal or variant factor IXa molecules or for factor IXN, resulted in a decrease in the Kd for factor IXaN to 0.67 nM, resulted in a suboptimal decrease in Kd for factor IXa AI (1.4 nM), and had no effect on the Kd for either factor IXa CH or factor IXN. The number of binding sites for factor IXa CH was not significantly different from that for factor IXa AI. The number of binding sites for factor IXaN was significantly lower than that for factor IXaN (p < 0.01) and was not significantly different from that for factor IXN.
presence of thrombin-stimulated platelets and factor VIII in the absence or presence of factor X. Gel-filtered platelets (3.5 × 10^6/ml) were incubated at 37 °C with human α-thrombin (0.1 unit/ml), CaCl_{2} (5 mM), and factor IXa, factor IXa_{H}, or factor IXa_{CH} in the presence or absence of thrombin-activated factor VIII (5 units/ml) and factor X (1.5 μM). Binding was determined as detailed under “Materials and Methods.” Nonspecific binding was determined in the presence of excess unlabeled factor IXa (0.44 nM) and was subtracted from total binding to obtain specific binding. The results shown represent a Scatchard plot of specific binding data for factor IXa in the absence (○) and presence (●) of factor VIIIa and factor X compared with specific binding data for either factor IXa_{H} (Δ, △) or factor IXa_{CH} (□, □) in the absence (Δ, □) or presence (△, △) of factor VIIIa and factor X. The plotted results represent mean values from six experiments with normal and variant factor IXa molecules, each done in duplicate.

**TABLE I**

| Ligand     | Factor VIII | Number of sites per platelet (±S.E.) | \( K_{d} \) (nM) | Equilibrium | Kinetic |
|------------|-------------|--------------------------------------|------------------|-------------|---------|
| Factor IXaN | Absent      | 540 ± 68                             | 2.3 ± 0.39       |             |         |
|            | Present     | 590 ± 60                             | 0.67 ± 0.05      | 0.61 ± 0.02 |         |
| Factor IXaAL | Absent   | 540 ± 52                             | 3.2 ± 0.41       |             |         |
|            | Present     | 506 ± 47                             | 1.4 ± 0.09       | 1.4 ± 0.10 |         |
| Factor IXaCH | Absent    | 331 ± 44                             | 2.8 ± 0.45       |             |         |
|            | Present     | 310 ± 45                             | 3.0 ± 0.51       | 2.0 ± 0.06 |         |
| Factor IXaN | Absent      | 306 ± 57                             | 2.6 ± 0.25       |             |         |
|            | Present     | 316 ± 50                             | 2.4 ± 0.30       |             |         |

**Kinetic Studies of Normal and Variant Factor IXa**—In this study we also determined the apparent \( K_{d} \) for normal and variant factor IXa binding to platelets by kinetic studies of factor IXa formation in the presence of saturating concentrations of factor X and factor VIIIa (Fig. 3). The kinetic approach gave similar results to the binding studies (3, 4), and its use is justified in our previous studies (3–5). The apparent \( K_{d} \) was determined as 0.61 nM for factor IXaN, 1.4 nM for FIXaAL, and 2.0 nM for FIXaCH (Fig. 3 and Table I).

We also determined the kinetic parameters for factor X activation by normal and variant factor IXa molecules in the presence of thrombin-stimulated platelets and factor VIIIa (Fig. 4). Studies were carried out at a factor IXa concentration of 0.01 nM, well below the apparent dissociation constant for binding of factor IXa to platelets. The values of \( k_{on} \), \( V_{max} \), \( h_{cat} \), and catalytic efficiency (\( k_{cat}/K_{m} \)) for factor IXaN, factor IXaAL, and factor IXaCH obtained at saturating concentrations of factor VIIIa are summarized in Table II. Catalytic efficiency can be assessed in two ways: 1) as a function of the total amount of factor IXa added, or 2) as a function of the amount of factor IXa bound to the platelet. If catalytic efficiency is assessed as a function of the amount of factor IXa bound, there is essentially no difference in the rate at which factor X is activated (the last column of Table II). So the decrease in the rate at which a given amount of factor IXaAL or factor IXaCH can catalyze factor X activation is solely due to the reduced affinity of these two proteins for the platelet-factor VIIIa-factor X complex.

**Fig. 3.** Rates of factor Xa formation by normal and variant factor IXa in the presence of thrombin-activated platelets and factor VIIIa. The rates of activation of human factor X by varying concentrations (0.17–5.0 nM) of factor IXa were determined in the presence of 3 × 10^6/ml thrombin-activated platelets at 37 °C in a reaction volume of 100 μl containing 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM CaCl_{2}, 1.5 μM factor X, 5 units/ml of factor VIIIa, and 0.5 mg/ml human serum albumin. Platelets were stimulated with 0.1 unit/ml thrombin in the presence of CaCl_{2} (5 mM), and factor IXa was preincubated with platelets for 10 min at 37 °C. Excess thrombin was neutralized with 50 nM PPACK before the addition of factor VIIIa and performance of the assay. For experimental details see “Experimental Procedures.” The plotted results for factor IXaN (●), factor IXaAL (▲), and factor IXaCH (■) are the mean ± S.E. of duplicate observations from five separate experiments.

**Fig. 4.** Factor X activation by normal and variant factor IXa. Initial rates of factor X activation (mean ± S.E.) were determined as described under “Experimental Procedures” at various concentrations of factor IXa as indicated in the graph. The experiment was carried out essentially as described in the legend to Fig. 3 except that factor X concentration was varied and the concentration of normal or variant factor IXa was 10 nM. The plotted results for factor IXaN (●), factor IXaAL (▲), and factor IXaCH (■) are the mean ± S.E. of duplicate observations from five separate experiments.
TABLE II

Binding and Kinetic Studies of Variant Factor IXa

| Normal and variant factor IXa catalyzed factor X activation: kinetic analysis |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | $K_m$ | $V_{max}$ | $k_{cat}$ | $k_{cat}/K_m$ | $V_{max}/K_m$ |
| Factor IXaA      | 0.10  | 18.7     | 1,870     | 2,671          | 18,700         |
| Factor IXaB      | 0.08  | 7.4      | 740.62    | 9,650          | 30,200         |
| Factor IXaC     | 0.18  | 12.5     | 1,250     | 6,621          | 6,944          |

$^a$ $k_{cat}$ expressed as moles of factor Xa per min per mol of total added factor IXa.
$^b$ $k_{cat}$ expressed as moles of factor Xa per min per mol of total platelet-bound factor IXa.


discussion

The purpose of the studies reported here was to begin an analysis of the structural features of the factor IXa molecule that are important for factor IXa binding to platelets and for the assembly of the factor X activating complex on the platelet surface. Previously we have shown that factor IXa binds reversibly to 500–600 sites per platelet and that platelet activation and the presence of calcium ions are required for this interaction (3). The dissociation constant ($K_d$) for factor IXa binding to activated platelets is $\sim 2.5 \text{ nM}$ in the absence of factor VIIIa and factor X and $\sim 0.5 \text{ nM}$ in the presence of these proteins at saturating concentrations (3). Similar findings have been reported for bovine aortic endothelial cells by Stern et al. (19). We have also shown that zymogen factor IX binds to 250–300 sites per activated platelet in the presence of calcium ions with a $K_d$ of $\sim 2.5 \text{ nM}$ either in the presence or absence of factors VIIIa and X and that factor IX competes with factor IXa for about one-half its low affinity sites in the presence of factors VIIIa and X (3). This suggests that the zymogen contains a domain important for binding of the enzyme to its platelet receptor and that the conversion of factor IX to factor IXa involves either a conformational alteration or the exposure of domains in factor IXa that allow it to interact with twice the number of platelet receptors as factor IX and also allow it to interact with factors VIIIa and X. We have further demonstrated that factor IXa binding to its high affinity site is closely correlated with rate enhancements of factor X activation by activated platelets (4), which can decrease the $K_m$ for factor X activation and can permit factor VIIIa to increase the $k_{cat}$ with a consequent increase of catalytic efficiency ($k_{cat}/K_m$) of $(17.4 \times 10^6)$-fold (5). Finally, we have studied the role of the active site of factor IXa in the binding of the enzyme to platelets by examining the interaction with platelets of factor IXa active site inhibited with danoyl L glutamyl glycyl L arginyl chloromethyl ketone (DEGR-CK). Since DEGR-factor IXa was shown to be a competitive inhibitor both of factor IXa binding and of factor X activation, with a $K_i$ almost identical with the $K_d$ for factor IXa binding, we concluded that the active site of factor IXa is not involved in binding to the high affinity site in the presence of factor VIIIa and factor X (4).

To determine the structural features of factor IXa that are required for interaction with platelet receptors and for assembly of the factor X activating complex, we studied the binding of factor IXaA and factor IXaC to activated platelets. It has previously been suggested (16, 20) that the abnormal conglutinating activities of these two proteins could be a consequence of deficient binding to charged membrane surfaces. However, it was subsequently shown (15) that the abnormal rates of factor X activation observed with both factor IXaA and factor IXaC in the presence of small, unilamellar vesicles composed of 30% phosphatidylethanolamine and 70% phosphatidylcholine were not a consequence of abnormal binding. Thus, the zymogen and activated forms of factor IXa, factor IXaA, and factor IXaC were shown to bind with similar affinities to small, unilamellar vesicles as determined by 90° light scattering (15). It was therefore concluded that the normal function of factor IXa must entail interactions between the light and heavy chains on the phospholipid surface.

The present studies addressed the structural requirements for binding of factor IXa to what must be presumed the physiologic locus of factor X activation, i.e. the platelet membrane. Factor IXaA was shown to bind with normal affinity ($K_d = 3.2 \text{ nM}$) to a normal number (540) of sites per platelet in the absence of factors VIIIa and X. This implies that the aspartic acid residue at position 47 in the normal protein (mutated to a glycine in factor IXaA) is not required for normal binding of factor IXa to its platelet receptor. This demonstration of a normal affinity of factor IXaA binding to platelet membranes in the absence of factors VIII and X confirms the results of Jones et al. (15) showing normal binding of factor IXaA to phospholipid membranes. However, in the presence of factors VIIIa and X, the binding affinity of factor IXaA was reduced ($K_d = 1.4 \text{ nM}$) as determined either by equilibrium binding or by kinetic determinations of rates of factor X activation (Table I). This result is not in conflict with those of Jones et al. (15) since their phospholipid binding studies were carried out only in the absence of factors VIII and X.

The results of our kinetic analysis of factor X activation by factor IXaA (Table II) help to clarify the interpretation of our binding studies and are also in general agreement with the previous factor X activation studies of Jones et al. (15). Thus, we found the $K_m$ for factor X activation by factor IXaA to be normal, suggesting that factor X binds with normal affinity to the factor IXaA-factor VIIIa-platelet membrane complex. The $V_{max}$ (7.4 nm·min$^{-1}$) with factor IXaA was 40% of normal, i.e. when compared with normal factor IXa (18.7 nm·min$^{-1}$), a result consistent with the reduction in relative rate of factor X activation by factor IXaA (43% of normal) obtained by Jones et al. (15) with phospholipids. In the present study, however, since we carried out both equilibrium binding studies in parallel with factor X activation studies, we were able to calculate true catalytic constants ($k_{cat}$) expressing the maximal rate of factor Xa catalysis as a function of the amount of factor IXa bound (Table II). The result of this analysis demonstrates a normal turnover number ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$) for factor IXaA compared with factor IXaB, indicating that the defect in factor X activation by factor IXaA is entirely a consequence of its decreased affinity for platelet receptors in the presence of factor VIIIa. Therefore, although the interpretation of the molecular basis for the defect is open to further study, it would appear that the Asp$^{47}$ → Gly mutation in factor IXaA resulted in a decreased affinity for platelet receptors in the presence of factor VIIIa and factor X.

A recent paper published by McCord et al. (21) reporting studies with factor IXaA provides evidence for a conformational change in factor IX due to high affinity calcium binding in the first epidermal growth factor domain. These authors found that although factor IXaA binds calcium ions normally to a high affinity site in the first epidermal growth factor domain, the variant enzyme fails to undergo a calcium-induced conformational change that occurs in normal factor IXa, thereby permitting it to interact properly with factor VIIIa and factor X. Thus, whereas factor IXaA was activated normally by factor XIa and factor IXaA had 52–60% of normal activity in a calcium/phospholipid vesicle system, the addition of factor VIIa decreased the relative rate of factor...
X activation by factor IXα₅₄₅₆ to 18–19% of normal. These observations are consistent with our suggestion that the defect in factor IXα₅₄₅₆ is a consequence of its failure to bind with normal affinity to membranes in the presence of factor VIIIa.

Our studies with factor IXα₅₄₅₆ show that this variant enzyme binds to platelets in a manner indistinguishable from normal zymogen factor IX (Table I). Thus, the number of binding sites per activated platelet appears to be similar to that for normal factor IX and about half the number for normal factor IXα. Moreover, the affinity of binding, which is 5-fold enhanced for normal factor IXα in the presence of factors VIIIa and X, is unaffected by factors VIIIa and X in the case of factor IXα₅₄₅₆. This very interesting result suggests that cleavage of factor IX at Arg'₄₅-Ala'₄₆ (defective in factor IXα₅₄₅₆ because of the Arg'₄₅ → His substitution), as well as at Arg'₈₀-Val'₈₁, with consequent formation of an activation peptide, is required for binding to the normal complement of receptors. In attempting to explain why the number of binding sites for factor IXα is almost exactly double that of zymogen factor IX, it is tempting to speculate that the receptor is bivalent (possibly homodimeric) and can accommodate two factor IXα molecules but only one factor IX molecule. It is possible that the presence of the heavily glycosylated activation peptide region of factor IX prevents access of a second factor IX molecule to the receptor complex, whereas formation of the activation peptide or its release from covalent attachment allows the resultant factor IXα to access both binding sites on the putative dimeric receptor. The fact that the affinity of factor IXα₅₄₅₆ for platelets is unaffected by the presence of factors VIIIa and X suggests that cleavage of factor IXα at Arg'₄₅-Ala'₄₆ is essential for the exposure in factor IXα₅₄₅₆ binding to platelets in the presence of factors VIIIa and X.

The present studies that demonstrate a normal affinity of factor IXα₅₄₅₆ binding to activated platelets in the absence of factors VIII and X are in agreement with the demonstration by Jones et al. (15) of normal binding of factor IXα₅₄₅₆ to phospholipid (phosphatidylserine/phosphatidylcholine) vesicles by 90° light scattering. Our demonstration that factor IXα₅₄₅₆ fails to bind to activated platelets with high affinity in the presence of factors VIIIa and X is not inconsistent with the phospholipid binding studies of Jones et al. (15) which were not done in the presence of factors VIII and X. Finally, the present studies demonstrating abnormally low rates of factor X activation by factor IXα₅₄₅₆ are entirely consistent with the studies of Jones et al. (15). Thus, as shown in Table II, the catalytic efficiency ($k_{cat}/K_m$) calculated for factor IXα₅₄₅₆ in the presence of activated platelets and factor VIIa was 6,944 μM⁻¹·min⁻¹ or 37% of normal (18,700 μM⁻¹·min⁻¹) when the $k_{cat}$ was based on the total amount of enzyme added, compared with a relative rate of factor X activation by factor IXα₅₄₅₆ shown to be 36% of normal by Jones et al. (15). However, when we calculated $k_{cat}$ as moles of factor Xa formed per mol of factor IXa bound, the catalytic efficiency proved to be essentially normal (36,783 min⁻¹ compared with 26,714 min⁻¹ for normal factor IXα). This indicates that the defect in factor X activation observed with factor IXα₅₄₅₆ is attributable solely to the decreased amount and affinity of factor IXα₅₄₅₆ binding to platelets in the presence of factors VIIIa and X.

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