Interaction of Factor IXa with Factor VIIIa

EFFECTS OF PROTEASE DOMAIN Ca\(^{2+}\) BINDING SITE, PROTEOLYSIS IN THE AUTOLYSIS LOOP, PHOSPHOLIPID, AND FACTOR X

(Received for publication, December 18, 1996, and in revised form, June 6, 1997)

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We previously identified a high affinity Ca\(^{2+}\) binding site in the protease domain of factor IXa involving Glu\(^{235}\) (Glu\(^{70}\) in chymotrypsinogen numbering; hereafter, the numbers in brackets refer to the chymotrypsinogen equivalents) and Glu\(^{245}\)[80] as putative ligands. To delineate the function of this Ca\(^{2+}\) binding site, we expressed IXa wild type (IXaWT), IXaE235K, and IXaE245V in 293 kidney cells and compared their properties with those of factor IX isolated from normal plasma (IXaNP); each protein had the same Mr, and \(\gamma\)-carboxyglutamic acid content. Activation of each factor IX protein by factor VIIa-Ca\(^{2+}\)-tissue factor was normal as analyzed by sodium dodecyl sulfate-gel electrophoresis. The coagulant activity of IXaWT was \(-95\%\), of IXaE235K was \(-27\%\), and of IXaE245V was \(-4\%\) compared with that of IXaNP. In contrast, activation by factor Xa-Ca\(^{2+}\) led to proteolysis at Arg\(^{158}\)-Ser\(^{159}\)[150–151] in the protease domain autolysis loop of IXaE245V with a concomitant loss of coagulant activity; this proteolysis was moderate in IXaE235K and minimal in IXaWT or IXaNP. Interaction of each activated mutant with an active site probe, p-aminobenzamidine, was also examined; the \(K_d\) of interaction in the absence and presence (in parentheses) of Ca\(^{2+}\) was: IXaNP or IXaWT \(230 \mu M (78 \mu M)\), IXaE235K \(150 \mu M (145 \mu M)\), IXaE245V \(225 \mu M (240 \mu M)\), and autolysis loop cleaved IXaE245V \(330 \mu M (350 \mu M)\). Next, we evaluated the apparent \(K_d\) (\(K_{app}\)) of interaction of each activated mutant with factor VIIIa. We first investigated the EC\(_50\) of interaction of IXaNP as well as of IXaWT with factor VIIIa in the presence and absence of phospholipid (PL) and varying concentrations of factor X. At each factor X concentration and constant factor VIIIa, EC\(_50\) was the free IXaNP or IXaWT concentration that yielded a half-maximal rate of factor Xa generation. EC\(_50\) values for IXaNP and IXaWT were similar and are as follows: PL-minus/X-minus (extrapolated), 2.8 \(\mu M\); PL-minus/X-saturating, 0.25 \(\mu M\); PL-plus/X-minus, 1.6 \(nM\); and PL-plus/X-saturating, 0.09 \(nM\). Further, \(K_{app}\) of binding of active site-blocked factor Xa to factor VIIIa was calculated from its ability to inhibit IXaWT in the Tenase assay. \(K_{app}\) values in the absence and presence (in parentheses) of PL were: IXaNP or IXaWT, 0.19 \(\mu M (0.07 \mu M)\); IXaE235K, 0.68 \(\mu M (0.26 \mu M)\); IXaE245V, 2.5 \(\mu M (1.35 \mu M)\); and autolysis loop-cleaved IXaE245V, 15.6 \(\mu M (14.3 \mu M)\). We conclude that (a) PL increases the apparent affinity of factor IXa for factor VIIIa \(-2,000\)-fold, and the substrate, factor X, increases this affinity \(-10–15\)-fold; (b) the protease domain Ca\(^{2+}\) binding site increases this affinity \(-15\)-fold, and lysine at position 235 only partly substitutes for Ca\(^{2+}\); (c) Ca\(^{2+}\) binding to the protease domain increases the S1 reactivity \(-3\)-fold and prevents proteolysis in the autolysis loop; and (d) proteolysis in the autolysis loop leads to a loss of catalytic efficiency with retention of S1 binding site and a further \(-8\)-fold reduction in affinity of factor IXa for factor VIIIa.

Factor IX is a vitamin K-dependent plasma protein that plays a crucial role in blood coagulation since the absence of its activity results in an X-linked bleeding disorder known as hemophilia B. The human protein is synthesized in the liver as a precursor molecule of 461 amino acids (1). The first 46 amino acids constitute the prepro leader sequence that is removed before secretion of the molecule. Also during biosynthesis, the protein undergoes several posttranslational modifications that include \(\gamma\)-carboxylation of first 12 Glu residues, partial hydroxylation of Asp\(_{\delta}\), and glycosylation at residues Ser\(_{\delta}\), Ser\(_{61}\), Asn\(_{125}\), Asn\(_{167}\), Thr\(_{159}\), and Thr\(_{169}\) (1–5). The resulting mature protein of 415 amino acids (Mr, 57,000) is a zymogen of serine protease factor IXa and contains 17% carbohydrate by weight (6).

Gene arrangement, amino acid sequence, and the x-ray structure of the protein strongly suggest that factor IX is organized into several distinct domains (1, 7). Circulating factor IX consists of an amino-terminal \(\gamma\)-carboxyglutamic acid (Gla) domain (residues 1–40), a short hydrophobic segment (residues 41–46), two epidermal growth factor (EGF)-like domains (EGF1 residues 47–84 and EGF2 residues 85–127), an activation peptide region (residues 146–180), and the carboxyl-terminal serine protease domain (residues 181–415). Based upon the crystal structure of the Gla domain of factor VIIa (8), the Ca\(^{2+}\) binding properties of factor X\(^{a}\), and the NMR structure of the Gla domain of factor IX (10), it would appear that this

\(^{a}\) This work was supported in part by National Institutes of Health Grant HL36363. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by a senior postdoctoral fellowship from the American Heart Association, Missouri Affiliate.

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1 The abbreviations used are: Gla, \(\gamma\)-carboxyglutamic acid; EGF, epidermal growth factor; TF, tissue factor; PL, phospholipid; S-2222, benzoyl-Asp\(_{\delta}\)-thrombin; WT, wild type; DEGR-ck, dansyl-Glu-Gly-Arg-chloromethyl ketone; BSA, fatty acid-free bovine serum albumin; p-AB, p-aminobenzamidine; NP, normal plasma; Ila, a-thrombin; TF, tissue factor; DEGR-IXa\(_{\delta}\), factor IXa inactivated with DEGR-ck; DEGR-IXa\(_{\delta}\), factor IXaWT inactivated with DEGR-ck; DEGR-IXa\(_{E235K}\), factor IXaE235K inactivated with DEGR-ck; DEGR-IXa\(_{E245V}\), factor IXaE245V inactivated with DEGR-ck; DEGR-IXa\(_{E245V}\), factor IXaE245V inactivated with DEGR-ck.

2 Sabharwal, A. K., Padmanabhan, K., Tulinsky, A., Mathur, A., Gorka, J., and Bajaj, S. P. (1997) J. Biol. Chem. 272, 22037–22045.
domain in factor IX possesses several low to intermediate affinity Ca$^{2+}$ binding sites. In addition, the EGF1 and protease domain each possess one high affinity Ca$^{2+}$ binding site (11, 12).

During blood coagulation, factor IX can be activated by factor VIIa-Ca$^{2+}$-tissue factor (TF) and by factor XIa-Ca$^{2+}$ (13). Activation by either enzyme occurs in two steps (6, 13). In the first step, the Arg$^{445}$-Ala$^{446}$ bond is cleaved which yields a two-chain disulfide-linked inactive intermediate called factor IXa$^\alpha$. In the second step, which is also the rate-limiting step (6, 13, 14), the Arg$^{180}$-Val$^{181}$ bond is cleaved giving rise to factor Ixa$^\beta$ (or simply factor IXa) and an activation peptide (AP). Factor Ixa$^\beta$ thus forms activates factor X in the clotting cascade. For maximal activation rate, this reaction requires Ca$^{2+}$, phospholipid (PL) and factor VIIa (15).

Existing evidence suggests that the Glu domain of factor IX binds to PL vesicles in the presence of Ca$^{2+}$ (16). The EGF1 domain of factor IX is required for its activation by factor VIIa-TF; in factor IXa, it may also interact with factor VIIa (17, 18). Moreover, the Ca$^{2+}$ binding site in the EGF1 domain appears to be necessary for its interaction with factor VIIa (17, 18). The role of the EGF2 domain is not clear, but it may be involved in protein-protein and protein-cofactor interactions (19). Finally, the protease domain is thought to play a primary role in binding to factor VIIa (20, 21). Although attempts have been made to investigate the role of the protease domain Ca$^{2+}$ binding site by mutational analysis, the data do not provide mechanistic details as to the inability of these mutants to function in clotting (22, 23). In this report, we have conducted a series of experiments to investigate the significance of the protease domain Ca$^{2+}$ binding site in factor IX function. Our data indicate that occupancy of this site in factor IXa results in maximal catalytic efficiency and factor VIIa binding. Further, proteolysis at Arg$^{178}$-Ser$^{179}$150–151 in the autolysis loop of factor IXa leads to a reduction in its affinity for factor VIIa binding, and this proteolysis is prevented by binding of Ca$^{2+}$ to the protease domain. An account of this work has been presented in abstract form (24).

**EXPERIMENTAL PROCEDURES**

Proteins and Reagents—Benzoyl-Ille-Glu-Gly-Arg-p-nitroanilide (S-2222) was purchased from Helena Laboratories. Dansyl-Glu-Gly-Arg-Dansyl-Glu-Gly-Arg-aminobenzamidine (p-AB) was purchased from Helena Laboratories. Factor IX was purchased from Human Protein Products. Human factor VIIa was a generous gift of Novo-Nordisk (Copenhagen). A monoclonal antibody-purified human factor VIII was obtained from Dr. Leon Hoyer (American Red Cross, Rockville, MD). The preparation was free of all other coagulation factors and contained human albumin as a stabilizing agent.

Coagulation Assay of Factor IX and Factor IXa—Factor IX and factor IXa activities were measured in a one-stage assay with automated partial thromboplastin time reagent as described (29). SDS-Gel Electrophoresis—SDS-gel electrophoresis was performed using the Laemmli buffer system (30). The acrylamide concentration was 7.5%, and the gels were stained with Commaassie Brilliant Blue.

Amino Acid Sequence Analysis—Automated Edman degradation of each protein component was performed using an Applied Biosystems 477A gas phase Sequencer. Approximately 0.2–0.5 nmol of protein was loaded on the filter cartridge. The proteins from SDS-gels were transferred to polyvinylidene difluoride membranes as described by Rosenberg (31).

Construction, Expression, and Purification of Recombinant Factor IXa Proteins—Wild type factor IX (IXWT), IXE235K (IX in which Glu$^{235}$ has been replaced by lysine), and IXE245V (IX in which Glu$^{245}$ has been replaced by valine) were constructed, expressed, and purified as described (17, 32).

Molecular Modeling—The putative model of the protease domain of human factor IXa was constructed using a homology model building approach described earlier (25). Crystallographic structure of the protease domain of porcine factor IXa in the absence of Ca$^{2+}$ was used as the starting template (7). The structure of trypsin and elastase provided the templates for the factor IXa region near the putative Ca$^{2+}$ binding site (33, 34).

p-AB Binding—Binding of p-AB was measured by an increase in its intrinsic fluorescence upon binding to the active site of each factor IXa protein using a Perkin-Elmer 650–605 fluorescence spectrophotometer. Details are given in a previous paper and in the legend to Fig. 3.

Preparation of DEGR-ck Inhibited Various Factor IXa Proteins—Each factor IX protein (200 mg/ml) was activated for 6 h by VIIaTF complex (2 mg/ml) in the presence of 1 ml PL vesicles in TBS, pH 7.4 (0.05 M Tris, 0.15 M NaCl, pH 7.4) containing 5 mM Ca$^{2+}$ SDS-gel electrophoretic analysis revealed full activation to IXa$^\beta$ without degradation to IXa$^\gamma$ forms. DEGR-IXa$^\beta$TP, DEGR-IXa$^\beta$TY, DEGR-IXa$^\beta$E245K, and DEGR-IXa$^\beta$E235K were prepared by adding 20-fold molar excess of DEGR-ck to each reaction tube. The pH was adjusted to 7.4, and each tube was incubated at 37 °C for 2 h. At this time, an additional 20-fold molar excess of the inhibitor was added, pH adjusted to 7.4, and the tubes were incubated for an additional 2-h period at 37 °C. Next, each tube again received 20-fold excess of the inhibitor; the samples were then incubated overnight at 4 °C, and the excess inhibitor was removed as follows. The samples were made 10 mM in EDTA and passed through Centricon 100 to remove the PL vesicles and relipidated TF. Free DEGR-ck was removed as described earlier (27, 35).

DEGR-IXa$^\gamma$E235K was prepared as follows. Factor IXaE235K was activated by TF as described in the legend to Fig. 3. DEGR-IXaE235K was prepared as above except four successive additions of the DEGR-ck were made instead of the three earlier; after the third addition, the tube was incubated for 2 h at 37 °C before the last addition and incubation overnight. Free DEGR-ck was removed as described earlier (27, 35). The absence of free DEGR-ck in our DEGR-IXa preparations was confirmed by the lack of their abilities to inhibit S-2222 hydrolysis by purified factor Xa. Moreover, when a known extinction coefficient (3.940 M$^{-1}$ cm$^{-1}$ at 340 nm) of the dansyl probe was used (36), we obtained stoichiometric (1.1 ± 0.05) incorporation of the inhibitor into each factor IXa protein.

Activation of Factor VIII by Ila—Except for reverse titration experiments (see Fig. 4), factor VIII at 40 units/ml was activated with 0.2 nm Ila in TBS/BSA, pH 8.0 (0.05 M Tris, 0.15 M NaCl, pH 8.0) containing 1 mg/ml BSA) and 5 mM Ca$^{2+}$. 10–15 ml aliquots were removed at 30-s intervals, diluted in cold TBS/BSA, 5 mM CaCl$_2$, pH 8.0, and assayed for factor VIII activity in a modified activated partial thromboplastin time assay. In this assay, 50 ml of hereditary factor VIII-deficient plasma was incubated with 50 ml of automated activated partial thromboplastin time reagent for 5 min at 37 °C. At this time, 50 ml each of 25 mM CaCl$_2$ and 0.5 mM DEGR-ck were added simultaneously and the clotting time noted. In initial experiments, it was found that factor VIII activity increased ~10-fold at 2 min, after which it declined steadily (32). Based upon these observations, we activated factor VIII with Ila for 2 min at which time Ila was inhibited by recombinant hirudin (2.2 nm, final concentration). The factor VIII sample was used immediately in the factor X activation experiments. In all experiments described in
paper, factor VIII activity units refer to those before activation with IIa. The functional molar concentration of IIa-activated factor VIII was determined by a technique described previously (37) for TF. The details are given in the legend to Fig. 4.

**Determination of Kd** of Factor IXa-Factor VIIIa Interaction in the Tenase Complex—These experiments were performed with IXaWT and IXaNP in both the absence and presence of PL vesicles. 50–μl reaction mixtures (in TBS/BSA, 5 mM CaCl2, pH 8.0) in the absence of PL vesicles were prepared containing various concentrations of factor IXaWT or factor IXαaNP (0–10 μM), a fixed concentration of factor VIIIa (20 units/ml prior to IIa activation), 7 and a constant concentration of factor X which was added last to initiate the reaction. The activation was carried out at 37 °C for 30–120 s at which time 1 μl of 0.5 M EDTA was added to stop further generation of factor Xa. A 40-μl aliquot was then added to a 0.1-ml quartz cuvette containing S-2222 in 75 μl of TBS/BSA, pH 8.0. The final concentration of S-2222 was 100 μM. The p-nitroaniline release was measured continuously (∆A405/min) for up to 20 min (27). Factor Xa was generated from a standard curve constructed using factor Xa prepared by insolubilized Russell’s viper venom. In control experiments, at each factor X concentration used, the reaction mixtures in the absence of Xa were prepared containing various concentrations of factor IXaWT or factor IXαaNP, and factor Xa generated was calculated from a standard curve which provided 50% of the Vmax enzyme kinetics program from Erithacus software (GraFit). To investigate the dependence of EC50 on factor X concentration, a series of such experiments was performed using several concentrations of factor X ranging from 15 nM to 5 μM. To obtain initial rates of factor Xa generation, less than 5% of factor X was allowed to activate in these experiments. Further, to prevent activation of factor X by the generated factor Xa (38), reactions were stopped before the formation of ~8 nM factor Xa in these experiments. This is based upon our observation that in these experiments factor Xa generation is linear with time only up to 10 nM, after which it increases with an upward slope.

The above experiments were also carried out in which the reaction mixtures contained 10 μM PL vesicles; this concentration of PL vesicles was chosen because it gave optimal rates of factor X activation (32). The concentration of factor VIIIa in these experiments was fixed at 0.1 unit/ml (before IIa activation), and the concentration of factor IXαaNP (NP or WT) ranged from 0 to 20 nM for each concentration of factor X. To obtain EC50 (functional Kd) values as a function of substrate concentration, a series of experiments was performed in which factor X was varied from 0.9 to 1.1 in all experiments indicating competition for a single binding site. The background value represented between 0.9 and 1.1 in all experiments indicating competition for a single binding site. The background value represented <5% of the maximum rate of Xa formation in the absence of DEGR-IXa.

**RESULTS AND DISCUSSION**

**Purification, Gla Content, NH2-terminal Sequence, and Activity of Factor IX Proteins—**SDS electrophoretic analysis of factor IX proteins using the Laemmli system (30) is shown in Fig. 1. Each protein is effectively homogeneous in this system (see zero time sample, Fig. 1). Plasma factor IX and each recombinant protein had similar Gla content (10.7–11.4 residues) as measured by the technique of Przybylski et al. (43). The amino-terminal sequence of each mutant protein was also

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5 One unit/ml of factor VIII after IIa activation contains ~0.7 μM factor IXa binding sites (see "Results and Discussion").
Our factor IXE235K preparation was consistently less than half that reported by Hamaguchi and Stafford (23), who found that lysine at position 250 (equivalent to 235 in factor IX) does not totally mimic the influence of Ca2+ on factor X structure and function. Further, our data indicate that occupancy of the Ca2+ site in the protease domain protects factor X mediated proteolysis of the Arg318-Ser319 peptide bond in the so-called autolysis loop (Fig. 2). Similarly, Ca2+ has been reported to inhibit significantly IIa-mediated proteolysis in the autolysis loop of plasma factor IX (45). Moreover, the autolysis loop-cleaved factor IXαE245V (present study) results in a complete loss of coagulant activity. To further these studies, we have used IXαE245V, IXαWT, IXαE235K, IXαβE235K, and IXαE245V, to investigate their binding to p-AB (availability of S1 site) and their abilities to compete for factor VIIIa in the Tenase complex (IXαVIIIα-Ca2+ + PL).

"p-AB Binding.—The data for p-AB binding are presented in Fig. 3 and summarized in Table 1. The fluorescence curve was observed to shift dramatically to the right in the absence of Ca2+ for IXαβNP (data not shown) with the Kd shifting from ~80 μM to ~230 μM. Our data for IXαNP are consistent with a previous report presented in abstract form (46). In contrast, the curves were identical for the mutants in the presence and absence of Ca2+. Moreover, the Kd of IXαβE245V was the same as IXαβNP in the absence of Ca2+, whereas it was ~150 μM for the IXαE235K. This again indicates that lysine at position 235 can only partially substitute for the Ca2+ site to maintain a native conformation. p-AB is known to bind to the S1 site of serine proteases. Thus, our data would indicate that binding of Ca2+ to the protease domain increases the reactivity of S1 site by ~3-fold.

Because IXαγE245V could be prepared easily by incubating IXαE245V with Xla-Ca2+ (see gel data presented later in Fig. 6A, inset), we also investigated its binding to p-AB. These data are presented in Fig. 3D. IXαγE245V bound p-AB with slightly reduced affinity (Kd ~340 μM) compared to Ca2+; however, the enhancement of the intrinsic fluorescence of p-AB upon binding to the S1 site was only ~25% of that observed with IXαβE245V. It should be noted that the enhancement of intrinsic fluorescence of p-AB was not observed when DEGR-IXαβE245V or DEGR-IXαγE245V was used in the p-AB titration experiments; this strongly indicates that the increase in intrinsic fluorescence observed with IXαγE245V is the result of the binding of p-AB at the active site. Because a reduced fluorescence increase was observed with IXαγE245V, it would indicate that the environment of the p-AB bound to the S1 site in IXαγE245V is less nonpolar compared with that in the IXαβE245V molecule. Based upon the work with other serine proteases (47), it would appear...
that Trp385[215] in factor IXa contributes to the nonpolar environment and therefore enhancement of p-AB intrinsic fluorescence upon binding at the active site; if so, then this region in factor IXaE245V is perturbed without the loss of S1 binding site. Such is also the case with factor Xa.

Recently, p-AB binding to factor IXa mutants lacking the protease domain Ca$^{2+}$ binding site has been reported (23); these authors performed all experiments in the presence of 5 mM Ca$^{2+}$. In their study, binding of p-AB to factor IXaE225SK caused an increase in the fluorescence intensity which was similar to the IXaWT. Moreover, only a small increase in the fluorescence intensity was observed in the case of either factor IXaE245K or factor IXaE225K&E245K (23). These observations can be rationalized based upon the data presented in Fig. 3. Previous measurements were made at a single concentration (150 μM) of p-AB and in the presence of 5 mM Ca$^{2+}$ (23). It is evident from Fig. 3 that this concentration is higher than the $K_d$ (~80 μM) for p-AB binding to factor IXaWT in the presence of Ca$^{2+}$ and is equal to the $K_d$ (~150 μM) for binding of p-AB to factor IXaE225SK in the presence or absence of Ca$^{2+}$. Thus, under these conditions (23), we estimate that 60 ± 10% of both factor IXaWT and factor IXaE225SK molecules will have p-AB bound at their active sites. As observed (23), this will result in a similar increase in fluorescence intensity for both of these proteins. In the case of factor IXaE225K (or IXaE225K&E245K), because factor XIa was used for activation, a significant proportion of each IXa protein could be proteolyzed in the autolysis loop and exhibit reduced fluorescence enhancement. Under the conditions of their experiments (23) and using the data of Fig. 3D, we calculate that for these mutants a fluorescence intensity change of 15–20% will be observed, an estimate that is close to the value reported.

**Influence of Factor X on the EC$_{50}$ of Interaction of IXaβ$_{NP}$ and IXaβ$_{NP}$ with Factor VIIIa**—For these experiments, we first wished to determine the concentration of active factor IXa binding sites in our IIa-activated factor VIII preparation. Two sets of experiments were performed for this purpose. Data were obtained with both IXaβ$_{WT}$ and IXaβ$_{NP}$. Because similar results were obtained with both proteins, only the data with the IXaβ$_{WT}$ are given. In one set of experiments, the factor IXa concentration was varied, and the factor VIII concentration was kept constant; before IIa activation, it was 0.1 unit/ml in the presence and 20 units/ml in the absence of PL. The rates of activation of factor X (2 μM in the presence and 3 μM in the absence of PL) were measured. These data for the IXaβ$_{WT}$ are presented in Fig. 4, A and B, in the presence and absence of PL, respectively. In a second set of experiments, the factor VIII concentration was varied, and the factor IXa concentration was kept constant; it was 0.025 nM in the presence and 15 nM in the absence of PL. Again, the rates of activation of factor X (2 μM in the presence and 3 μM in the absence of PL) were measured. These data for IXaβ$_{WT}$ are presented in Fig. 4, C and D, in the presence and absence of PL, respectively. Values of $V_{max}$ were calculated using the enzyme kinetics program from Erithacus software. The concentrations of factor VIII in molar terms were calculated using the following equation,

\[
\text{VIII concentration, nM units/ml} = \frac{V_{max1}}{V_{max2}} \times \frac{\text{IXa, nM}}{\text{VIII, units/ml}} \quad (\text{Eq. 4})
\]

where $V_{max1}$ is the rate of factor X activation at a constant concentration of IIa-activated factor VIII (Fig. 4, A or B) and $V_{max2}$ is the rate of factor X activation at a constant concentration of IXaβ$_{WT}$ (Fig. 4, C or D). Using Equation 4 and the data of Fig. 4, A and C, we calculate that 1 unit/ml factor VIII clotting activity (before IIa activation) corresponds to 0.65 nM. Similarly, from the data of Fig. 4, B and D, 1 unit/ml factor VIII corresponds to 0.75 nM. Thus, 1 unit/ml factor VIII after IIa activation contains ~0.7 nM factor IXa binding sites as measured in the Tenase assay system with or without PL.

The nanomolar concentrations of factor VIIIa when indicated in this paper are based upon the above calculation. Here, we wish to point out that factor VIII after cleavage by thrombin is not a stable protein, and therefore all studies of factor IXa-factor VIIIa interaction are complicated by this inherent instability of factor VIIIa. This is complicated further by the fact that the unstable factor VIIIa molecule is, in part, stabilized by complexation with factor IXa and PL vesicles (48). Moreover, potential also exists that factor IXa at high concentrations may
slowly proteolyze factor VIIIa, leading to further losses in factor VIIIa activity (49). Therefore, determinations of EC50 values (functional $K_d$) and $K_{app}$ values of IXa-VIIIa interaction presented below should be interpreted with this caveat in mind. However, one should note that the knowledge of absolute concentrations of factor VIIIa is not critical to the conclusions drawn from this paper.

Next, we measured the EC50 of interaction of IXa with factor VIIIa. These data are presented in Fig. 5 and summarized in Table II. Clearly the EC50 value is influenced by the amount of factor X present in the reaction mixture in a PL-free system as well as in a PL-containing system. In the absence of PL, the EC50 of IXa-factor VIIIa interaction at saturating concentrations of factor X is $-0.25 \mu M$, and an extrapolated value in the absence of factor X is $-2.8 \mu M$. In the presence of PL, the EC50 of IXa-factor VIIIa interaction at saturating concentrations of factor X is $-0.09 \mu M$, and an extrapolated value in the absence of factor X is $-1.6 \mu M$. Thus, factor X decreases the EC50 (functional $K_d$) by an order of magnitude both in the presence or absence of PL, whereas PL decreases this value by $-2,000$-fold in either the presence or absence of factor X.

In an earlier study (39), it was reported that the factor IXa-factor VIIIa interaction is not influenced by the presence of factor X. However, in that study the total concentration of factor IXa used in the factor VIIIa titration experiment was $-19 \mu M$, which is substantially higher than the functional $K_d$ of $-2 \mu M$ or EC50 value of $-0.09 \mu M$ (present study). Use of such high concentrations of factor IXa ligand can lead to large inaccuracies in measuring $K_d$ values in the picomolar range and obscure the effect of factor X. Our data using a wide range of factor X concentrations clearly demonstrate that the EC50 (functional $K_d$) of the factor IXa-factor VIIIa interaction is dependent upon the concentration of the substrate, factor X. The functional $K_d$ of the interaction of factor VIIa with TF has also been reported to be dependent upon the substrate concentration (50). However, it should be noted that the EC50 (functional $K_d$) value for the factor Xa-factor Va interaction is not dependent upon the prothrombin concentration in the prothrombinase system (51). An explanation for these observations might be that factor X (or another substrate) binding to factor IXa or factor VIIa locks each enzyme into a favorable...
conformation for interaction with factor VIIIa or tissue factor, respectively. In contrast, factor Xa might exist in a conformation, which is optimal for interaction with factor Va even in the absence of prothrombin.

Our data on the effect of PL in increasing the interaction of factor IXa with factor VIIIa are consistent with the observations made with other PL-dependent enzyme-cofactor interactions (27, 36, 50, 52, 53). In the presence of PL vesicles, the $K_d$ of the interaction of factor VIIa with TF decreases from $\sim 1-10$ nM to $\sim 5-10$ pM (27, 36, 50), and the $K_d$ of the interaction of factor Xa with factor Va decreases from $\sim 1 \mu M$ (52, 53) to $\sim 30$ pM (52). The effect of PL in all cases may be attributable to restricting the rotational and translational diffusion of the PL-bound proteins involved in interaction with each other (54). Overall, the data indicate that in the presence of PL, all three enzymes (factors VIIa, IXa, and Xa) bind to their respective cofactors (tissue factor for VIIa, VIIIa for IXa, and Va for Xa) with apparent dissociation constants in the low picomolar range.

**Measurements of Apparent $K_d$ Values for the Interaction of Each Factor IXa Species with Factor VIIIa in the Tenase Complex**—Factor XIa-mediated activation of factor IX mutants lacking the protease domain Ca$^{2+}$ binding site results in proteolysis in the autolysis loop with concomitant loss of coagulant activity (Fig. 2). Furthermore, although these mutants could be activated normally without proteolysis in the autolysis loop by VIIa-TF (Fig. 1), we were unable to remove VIIa-TF completely from the activation mixtures. Since even minuscule amounts of contaminating VIIa-TF complex can contribute significantly to factor X activation, especially in the case of mutants, it precluded our measurements of EC$_{50}$ values (functional $K_d$) for the interaction of each activated mutant with factor VIIIa. However, each preparation of factor IXa protein after inhibiting with DEGR-ck was found to have no VIIa-TF activity as measured by its ability to activate factor X. To determine the $K_d$ values of interaction of these active site-blocked mutants with factor VIIIa, we evaluated their abilities to compete with factor IXa$_{WT}$ (prepared by factor XIa activation) in binding to factor VIIIa in the Tenase complex. Because a mutation in factor IXa could alter factor VIIIa binding by at least two mechanisms (by perturbation of the contact site and by spatial misalignment of the otherwise normal contact site above the PL surface) we investigated the inhibition of Tenase activity by these mutants in the presence and absence of PL vesicles.

The steady-state inhibition curves (55) obtained in the presence (Fig. 6A) and absence (Fig. 6B) of PL were analyzed as outlined under “Experimental Procedures.” The $K_{d,app}$ values for the interaction of active site-blocked mutants with factor VIIIa are listed in Table III. DEGR-IXa$_{E245V}$ and DEGR-IXa$_{E235K}$ interacted with factor VIIIa with a $K_d$ of $\sim 70$ pM; this value of $K_d$ is close to the estimated EC$_{50}$ value ($\sim 90$ pM) obtained at saturating concentrations of factor X. Because the DEGR moiety in factor IXa$\beta\beta'$ is expected not to participate in direct binding to factor VIIIa, it supports the concept that the increase in the affinity of factor IXa$\beta\beta'$ is the result of a conformational change induced by occupancy of the active site, either by DEGR-ck or by factor X. Our $K_{d,app}$ value ($\sim 70$ pM) of DEGR-IXa$_{E245V}$ interaction with factor VIIIa is much lower than the value ($\sim 2$ nM) obtained by fluorescence anisotropy measurements (39). As pointed out earlier, this difference may be attributable to the $\sim 300$-fold higher concentration (above the $K_{d,app}$ value) of active site-blocked factor IXa used in previous experiments.

The binding of factor VIIIa to each active site-blocked mutant was considerably weaker both in the presence and absence of PL vesicles (Table III). Compared with DEGR-IXa$_{E245V}$, DEGR-IXa$_{E235K}$ had similarly (−4-fold) reduced affinity for factor VIIIa in the presence or absence of PL. However, DEGR-IXa$_{E245V}$ had $\sim 20$-fold reduced affinity in the presence of PL and $\sim 13$-fold reduced affinity in the absence of PL, whereas DEGR-IXa$_{E235K}$ had $\sim 200$-fold reduced affinity in the presence of PL and $\sim 80$-fold reduced affinity in the absence of PL. These data indicate that in the case of DEGR-IXa$_{E245V}$ or DEGR-IXa$_{E235K}$, in addition to the perturbation of factor VIIIa binding site, a further reduced affinity in the presence of PL could, in part, be due to the misalignment of the factor VIIIa contact site. Importantly, these data indicate that the protease domain Ca$^{2+}$ binding site is essential for stabilizing the native conformation of this domain needed for factor VIIIa binding.
from this and reactivity of the S1 site by this Ca2+

and lysine at position 235 cannot fully mimic the function of IIa. However, it should be noted that the Ca2+

may not directly participate in factor VIIIa binding (58). The spatial arrangement of Ca2+ binding site, autolysis loop, and S1 site is depicted in Fig. 7. An examination of this figure would indicate that binding of Ca2+ in this domain allosterically affects the factor VIIIa as well as the S1 binding site. Further, binding of factor VIIIa to this domain appears to alter the conformation of the active site (9) with a resultant increase in the kcat for factor X activation (15).

Acknowledgments—We thank Dr. K. Padmanabhan (Department of Biochemistry, Michigan State University, East Lansing) for help in modeling the protease domain of human factor IXa and Beth Haase for preparing the manuscript.

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