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Articles

Role of Calcium Ions and the Heavy Chain of Factor XIa in the Activation of Human Coagulation Factor IX†

Dipali Sinha,*,‡ Frances S. Seaman,‡ and Peter N. Walsh‡,§

Thrombosis Research Center, Department of Medicine, and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

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ABSTRACT: Since optimal rates of factor IX activation by factor XIa require the presence of calcium ions and the heavy chain of the enzyme as well as the active-site-containing light chain, we have studied the effects of calcium ions and the heavy chain on the reaction kinetics. Whereas the amidolytic activities of factor XIa and of its active-site-containing light chain were almost indistinguishable, the two enzymes behaved quite differently when factor IX was the substrate. Factor XIa was 100-fold more potent in the presence of Ca^{2+} than in its absence. On the contrary, the presence or absence of Ca^{2+} made very little difference in the case of the isolated light chain of factor XIa. Moreover, the enzymatic activity of the light chain was almost identical with that of intact factor XIa when Ca^{2+} was absent. Using an optimal concentration of Ca^{2+}, we studied the activation in the presence of various concentrations of two monoclonal antibodies, one (5F4) directed against the light chain of factor XIa and the other (3C1) against its heavy chain. Analysis of $1/V$ vs. $1/S$ plots showed that whereas inhibition by 5F4 was noncompetitive, 3C1 neutralized the enzyme in a classical competitive fashion. We conclude that in the calcium-dependent activation of factor IX by factor XIa the heavy chain of the enzyme is involved in the binding of the substrate and this is essential for optimal reaction rates.

Factor XI is a coagulation protein present in human plasma in zymogen form at a concentration of 4-6 μg/mL. Since a hemorrhagic state can result from its deficiency, it undoubtedly has a key role in the regulation of blood coagulation (Rosenthal et al., 1953). Factor XI migrates with an apparent molecular weight of 160 000 on sodium dodecyl sulfate ($NaDodSO_4$)[1] gels and consists of two identical disulfide-linked polypeptide chains (Koide et al., 1977; Kurachi & Davie, 1977; Bouma & Griffin, 1977). When activated by factor XIIa, an internal peptide bond in each of its two polypeptide chains is cleaved giving rise to a pair of disulfide-linked heavy and light chains with molecular weights of 50 000 and 30 000, respectively, each light chain containing one active site (Kurachi & Davie, 1977; Bouma & Griffin, 1977). Factor XIa is the activator of factor IX in the intrinsic pathway of blood coagulation (Davie et al., 1979; Fujikawa et al., 1974). The rate of activation of factor IX by factor XIa is greatly increased in the presence of calcium ions. Factor IX activation by factor XIa involves a two-step

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* Address correspondence to this author c/o Dr. Peter N. Walsh, Thrombosis Research Center, Temple University School of Medicine.
‡ Thrombosis Research Center, Department of Medicine, Temple University School of Medicine.
§ Department of Biochemistry, Temple University School of Medicine.

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; TBS, Tris-buffered saline (50 mM Tris/100 mM NaCl, pH 7.5); BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; pNA, *p*-nitroanilide; TCA, trichloroacetic acid; RIA, radioimmunoassay.

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mechanism. In the first step, an Arg–Ala bond is cleaved, giving rise to a two-chain intermediate which is further cleaves at an Arg–Val bond in the second step resulting in the release of an activation peptide (M_r ~11 000) and the formation of active factor IXa (Fujikawa et al., 1974; Osterud et al., 1978). It has been reported previously that the isolated light chain of factor XIa, which retains its full enzymatic activity against a synthetic tripeptide substrate (pyro-Glu-Pro-Arg-pNA or S-2366), has considerably less enzymatic activity against its natural substrate factor IX, suggesting a role of the heavy chain of factor XIa in the activation of factor IX (van der Graaf et al., 1983; Sinha et al., 1985). Using monoclonal antibodies directed against different epitopes of factor XIa, we have studied the reaction rates and have shown that in the activation of factor IX by factor XIa an interaction of factor IX with the heavy chain of factor XIa is probably essential for the efficient cleavage of the substrate (Sinha et al., 1985). These studies were made in the presence of 5 mM calcium which is known to enhance the rate of the reaction. However, the mechanism by which calcium ions enhance the rate of the reaction is not known. The present project was undertaken to determine whether calcium ions have a role in the interaction of factor IX with the heavy chain of factor XIa. We have, therefore, studied both the calcium-dependent and the calcium-independent activation of factor IX by factor XIa and by the isolated light chain. We have also determined the kinetic parameters of the activation reaction in the presence and absence o. monoclonal antibodies specific for the light chain and the heavy chain of factor XIa.

Experimental Procedures

Materials. All chemicals were the best grade commercially available and were purchased from Sigma Chemical Co., St. Louis, MO; Fisher Chemical Co., Fairlawn, NJ; or J. T. Baker Chemical Co., Phillipsburg, NJ. Plasmas deficient in coagulation factors were purchased from George King Biochemical, Overland Park, KS. Betaphase scintillation fluid was obtained from West Chem Products, San Diego, CA. Tritiated sodium borohydride (75 Ci/mmol) was purchased in crystalline form in sealed ampules from New England Nuclear, Boston, MA. Crystallized bovine serum albumin (BSA), benzamidine hydrochloride, soybean trypsin inhibitor, sodium heparin, and cephalin (rabbit brain extract) were purchased from Sigma Chemical Co. (St. Louis, MO). Carrier-free $Na^{125}I$ was obtained from New England Nuclear. The chromogenic substrate pyro-Glu-Pro-Arg-*p*-nitroanilide dihydrochloride (S-2366) was a gift from Kabi Peptide Research (Stockholm, Sweden).

Purification of Proteins. Factor XI was purified from 2 L of human plasma by a modification (Sinha et al., 1984) of the method of Bouma and Griffin (1977). The purified protein appeared homogeneous on polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ as previously published (Sinha et al., 1984) and had a specific activity of 270 units/mg of protein. It was stored in 0.2 M sodium acetate/0.6 M NaCl, pH 5.3 at −70 °C. Human factor IX was purified to a specific activity of 225 units/mg by a modification (Walsh et al., 1984) of methods described by DiScipio et al. (1977) and by Miletich et al. (1980) and was a single band on $NaDodSO_4$ gel electrophoresis as previously published (Walsh et al., 1984). Bovine two-chain factor XIIa was kindly provided by Dr. E. P. Kirby of the Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA, as a homogeneous protein which hydrolyzed 3.78×10^9 mol of the chromogenic substrate S-2302 min^{-1} (μg of protein)$^{-1}$ at pH 8.0 and 37 °C. Details of the production and purification of monoclonal antibodies against factor XI have been previously published (Sinha et al., 1985).

Preparation and Characterization of Factor XIa. Purified factor XI was activated by incubation at 37 °C with bovine factor XIIa as previously described (Sinha et al., 1984). To 100 μL (100 μg) of purified factor XI (in 0.2 M acetate buffer, pH 5.3), concentrated Tris base was added to adjust the pH to 7.5, and this was followed by incubation with 6 μL (3 μg) of two-chain bovine factor XIIa at 37 °C for 3–4 h. Gel electrophoresis in the presence of $NaDodSO_4$ of this preparation under reducing conditions showed two major bands of M_r 50 000 and 30 000, respectively.

Radiolabeling of Proteins. Factor IX was labeled with tritium by a previously described modification (Walsh et al., 1984) of the method described by Van Lenten and Ashwell (1971) and adapted for bovine factor X by Silverberg et al. (1977). The labeled protein was finally purified by alkaline gel electrophoresis and appeared homogeneous as judged by $NaDodSO_4$ gel electrophoresis and fluorography as previously reported (Walsh et al., 1984), and >98% of the radioactivity was precipitable in 5% trichloroacetic acid. The tritiated factor IX had a specific radioactivity of 475 cpm/ng and retained >90% of its coagulant activity compared with unlabeled factor IX. Affinity-purified goat anti-mouse IgG (Pel-Freez, Rogers, AR) was radiolabeled by a minor modification of the Iodogen method (Fraker & Speck, 1978) to a specific activity of 4.45 μCi/μg.

Protein Analyses. Protein concentrations were determined by the Bio-Rad dye binding assay according to instructions provided by the manufacturer (Bio-Rad, Richmond, CA). Purified monoclonal antibodies were quantitated by using an extinction coefficient of 14 for a 1% solution at 280 nm. Polyacrylamide slab gel electrophoresis in $NaDodSO_4$ was done by the procedure of Laemmli (1970).

Coagulation Assays. Factor IX was assayed by utilizing minor modifications of the kaolin-activated partial thromboplastin time (Proctor & Rapaport, 1961) using factor IX deficient substrate plasma. The procoagulant activities of factor XI and factor XIa and its light chain were determined as follows: 100 μL of factor XI deficient plasma was incubated with 100 μL of kaolin (5 mg/mL in saline), 100 μL of 0.2% inosithin in 20 mM Tris/saline, pH 7.4, and 10 μL of plasma or sample + 90 μL of the above-mentioned buffer for 5 min at 37 °C. Then 100 μL of 30 mM $CaCl_2$ was added to initiate clot formation. The observed clotting times were converted to clotting units by comparison to the clotting activities of serial dilutions of a normal pooled plasma.

Effects of Monoclonal Antibodies on the Rate of Activation of Factor IX by Factor XIa or Its Light Chain in the Presence and Absence of Calcium. The rate of activation of ^3H-labeled factor IX was followed by measurements of the trichloroacetic acid soluble activation peptide released during enzymatic cleavage by factor XIa or by its light chain (Walsh et al., 1984). Either factor XIa or the isolated light chain of factor XIa, prepared as previously described (Sinha et al., 1985), was incubated for 20 min at 37 °C either with buffer or with antibody solution before addition to the incubation mixture which contained ^3H-labeled factor IX. The release of ^3H-labeled activation peptide in 5% TCA was measured essentially as described previously (Walsh et al., 1984). Assays were carried out in Tris (50 mM)/NaCl (100 mM), pH 7.5 (TBS), in a total volume of 300–400 μL containing 5 mg/mL bovine serum albumin (BSA). At various time intervals, 40-μL samples were removed and added to 120 μL of an ice-cold mixture containing 1 part TBS and 2 parts 50 mM EDTA,

pH 7.5. To this reaction mixture was added 80 μL of ice-cold 15% TCA, which was then vortexed vigorously followed by centrifugation at 10000*g* for 3 min in a benchtop Brinkmann Model 3200 microfuge (Brinkmann Instruments, Inc., Westbury, NY). Tritium in the supernatant was counted in a Beckman LS 8000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA). When activation rates were studied in the presence of Ca^{2+}, the assay mixture was made 5 mM with respect to $CaCl_2$ concentration. To study activation rates in the absence of calcium, two sets of experiments were performed: one in which there was no added calcium in the assay mixture and the other in which it was made 2 mM in EDTA. No differences were observed without added Ca^{2+} or with EDTA, and all subsequent experiments designated as being done without Ca^{2+} were carried out in the presence of 2 mM EDTA. When the activation was studied in the absence of calcium, the concentration of ^3H-labeled factor IX and that of the light chains of factor XIa were the same as for the activation in the presence of calcium, whereas the active-site concentration of factor XIa was 10 times higher.

Kinetics of Activation of Factor IX by Factor XIa and by the Light Chain of Factor XIa. The assay procedure was the same as described above except that the initial rates of activation were determined over a wide range of substrate concentrations using a mixture of 1 part ^3H-labeled factor IX and 9 parts unlabeled factor IX as described previously (Walsh et al., 1984). When the experiments were performed in the absence of calcium, the reaction mixture was made 2 mM in EDTA to make sure that any residual calcium from buffers or protein preparations was chelated. For determination of the effects of the antibodies on the kinetics of the reaction in the presence of Ca^{2+}, factor XIa was preincubated with various concentrations of the antibody for 20 min at 37 °C before being added to the assay mixture. When the activation was performed at various concentrations of Ca^{2+}, the dose–response curves in the presence and absence of the antibody were exactly identical, both showing an optimal Ca^{2+} concentration in the range of 2–5 mM (data not shown). Therefore, for the determination of the kinetic parameters at various concentrations of the antibodies, all the experiments were done in the presence of 5 mM Ca^{2+}. The initial rates of release of the activation peptide were linear and were determined under conditions where less than 10% of the substrate had been consumed.

Calculation of Kinetic Constants. The derivation of kinetic constants for factor IX activation by factor XIa was based on a one-enzyme, one-substrate model. Values for the Michaelis constant (K_m) and the maximum velocity (V_{max}) were obtained by the Lineweaver–Burk method (Segal, 1974a) and were calculated by using least-squares fit and the FORTRAN program of Cleland (1967) with a TRS-80 computer. Values of kinetic constants obtained by the Cleland program and by graphical analysis were in close agreement (i.e., within 10%).

Kinetics of Hydrolysis of the Synthetic Substrate Pyro-Glu-Pro-Arg-pNA (S-2366) by Intact Factor XIa and by Its Isolated Light Chain. The amidolytic assay of factor XIa or its light chain using the oligopeptide substrate pyro-Glu-Pro-Arg-pNA was done by a modification of the procedure described earlier (Scott et al., 1984). Factor XIa or the isolated light chain of factor XIa at a final concentration of 1 nM was added to a total volume of 300 μL of Tris (0.05 M)/NaCl (0.10 M), pH 7.5, containing various concentrations of the substrate ranging from 0.74 to 0.55 mM. The initial rate of hydrolysis of duplicate samples was measured spectrophotometrically at 405 nm with a Gilford System 2600 spectrophotometer (Gilford, Oberlin, OH).

Determination of the Binding Constants of Antibodies to Factor XI and Factor XIa. Solid-phase radioimmunoassay (RIA) was performed essentially as described by Frankel and Gerhard (1979) to determine the binding constants of the two monoclonal antibodies 5F4 and 3C1 to factor XI. Factor XI or factor XIa was bound to the wells of a 96-well polyvinyl microtiter plate (Dynatech, Alexandria, VA) by incubation with 100 μL of the protein solution (10 μg/mL) overnight at 4 °C. After the remaining slices of the wells were blocked by incubation with 200 μL of Tris (0.05 M)/NaCl (0.10 M) containing 5 mg/mL BSA for 2 h at room temperature, 100 μL of the antibody solution was added and incubated overnight at 4 °C. The plate was then thoroughly washed to remove any unbound antibody. One hundred microliters of ^{125}I-labeled affinity-purified goat anti-mouse IgG was then added to each well and incubated for 4 h at room temperature. After the wells were thoroughly washed, they were separated from the plate and counted in a γ counter (Nuclear Enterprises, NE 1600, Scotland, U.K.). In order to determine the effect of Ca^{2+} on the solid-phase RIA, two identical sets of experiments were performed, one in which all the buffers were made 5 mM in Ca^{2+} and the other in which there was no added Ca^{2+}.

For the determination of binding constants, a preliminary experiment was first performed in which one antibody concentration was chosen and titrated with increasing amounts of antigen coating a series of wells such that a condition was determined whereby all of the added antibody was bound to the antigen. Thus, knowing the number of cpm of ^{125}I-labeled goat anti-mouse IgG retained in a given well, the amount of the primary antibody bound to the antigen could be related to the number of cpm of ^{125}I-labeled goat anti-mouse IgG retained. Employing conditions thereby defined, the amount of antibody bound could be determined when serial dilutions of the antibody solution were added to wells containing a constant amount of antigen. Since the total amount of antibody added was known, and the amount bound bould be determined, the amount free could be calculated and the binding constant could be determined as described by Scatchard (1949):

$$[A]_{bound}/[A]_{free} = sK[P]_{total} - nK[A]_{bound} \quad (1)$$

where n = the valence of antibody A, s = the valence of antigen P, and K = the binding constant of the reaction.

Analysis of Kinetic Data for the Quantitation of the Inhibitor Constants. Since the antibodies used as inhibitors bind to factor XIa relatively tightly (see Results), we used the I_{50} method developed by Cha (1975) to determine the inhibitor constants. In the case of classical competitive inhibition, I_{50} (total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction) is related to the substrate concentration as follows:

$$I_{50} = \tfrac{1}{2}E_t + K_i + K_iS/K_m \quad (2)$$

where E_t = the total enzyme concentration and S = the substrate concentration. Thus, from the plot of I_{50} vs. S, K_i (the inhibitor constant) can be determined.

In the case of noncompetitive inhibition, I_{50} is independent of the substrate concentration and related to K_i as follows:

$$I_{50} = \tfrac{1}{2}E_t + K_i \quad (3)$$

K_i can be evaluated by determining I_{50} at several enzyme concentrations.

RESULTS

Comparison of the Effects of the Antibodies on the Activation of Factor IX by Factor XIa or by Its Light Chain in

Table I: Functional Comparison of Factor XIa and Its Light Chain[a]

enzyme	amidolytic act. [μmol of pNA released (μmol of enzyme)$^{-1}$ s^{-1}]	antibody	initial rate of factor IX activation [mol of ^{3}H-peptide released (mol of enzyme)$^{-1}$ min^{-1}] in the presence of Ca^{2+}	in the absence of Ca^{2+}
factor XIa	173		3.4	0.038
		5F4	0.076	0.0009
		3C1	0.53	0.053
light chain	168		0.037	0.0093
		5F4	0.00096	<0.0005
		3C1	0.034	0.0093

[a] The amidolytic assay was performed as described under Experimental Procedures and as reported previously (Scott et al., 1984). Details of the procedure for factor IX activation are described in the text. The initial rates of factor IX activation were calculated from Figure 1, as described in the text.

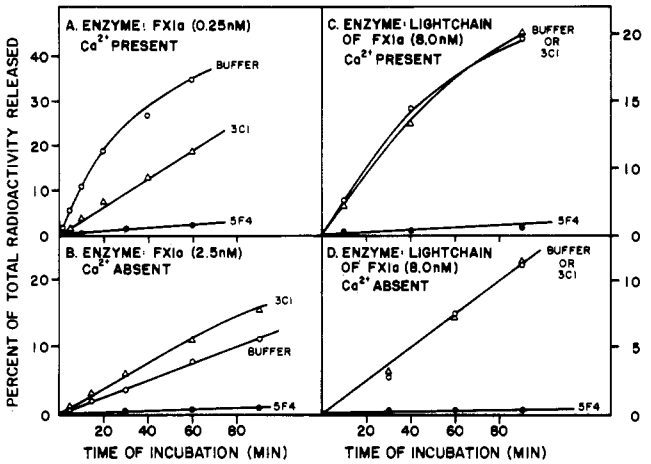

FIGURE 1: Effect of heavy chain specific (3C1) and light chain specific (5F4) monoclonal antibodies against factor XIa on the activation of factor IX. The same concentration of ^{3}H-labeled factor IX (35 nM) was used in all four sets of experiments. The molar ratio of factor XIa or the light chain of factor XIa to 5F4 was 1:5 and 1:10 for 3C1. Results shown are (○) in the absence of antibody, (●) in the presence of 5F4, and (△) in the presence of 3C1. (A) Activation by factor XIa (0.25 nM) in the presence of $CaCl_2$ (5 mM). (B) Activation by factor XIa (2.5 nM) in the absence of Ca^{2+}. (C) Activation by the light chain of factor XIa (8.0 nM) in the presence of $CaCl_2$ (5 mM). (D) Activation by the light chain of factor XIa (8.0 nM) in the absence of Ca^{2+}.

the Presence or Absence of Calcium Ions. The rate of activation of factor IX by factor XIa in the presence and absence of the antibodies was studied over a range of concentrations of factor IX (2–20 μg/mL), factor XIa (0.01–0.05 μg/mL), and antibodies (0.05–1.0 μg/mL). The observations shown in Figure 1 and Table I are typical of those experimental determinations.

The effects of the antibodies on the rate of activation of factor IX by the light chain of factor XIa in the presence of calcium are shown in Figure 1C whereas those in the absence of calcium are shown in Figure 1D. The light chain specific antibody 5F4 inhibited the rate of activation almost completely both in the presence and in the absence of calcium. On the other hand, the heavy chain specific antibody 3C1 did not have any effect either in the presence or in the absence of calcium. Figure 1A,B illustrates the effects of the same two antibodies on the activation of factor IX when intact factor XIa was used as the enzyme. The light chain specific antibody 5F4 inhibited the rate of activation both in the presence and in the absence of calcium exactly the same way as observed in the case of the activation by the light chain. However, the effect of the heavy chain specific antibody 3C1 was dramatically different. In the presence of calcium, 3C1 inhibited the rate of activation of factor IX by intact factor XIa whereas in the absence of calcium the inhibitory effect of 3C1 was totally abolished; in fact, there was a slight stimulatory effect. Even at 10-fold higher concentration of 3C1 than reported here, no inhibitory effect was observed when the activation was carried out in the absence of Ca^{2+}. In control experiments, normal mouse IgG did not have any significant effect on the rate of activation of factor IX by factor XIa as reported earlier (Sinha et al., 1985). The binding of factor XI and of factor XIa to the antibodies, measured by solid-phase radioimmunoassay, was also found to be identical in the presence and absence of Ca^{2+} (data not shown).

Table II: Kinetic Parameters of Factor IX Activation under Various Experimental Conditions

experimental conditions	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
intact factor XIa, 5 mM Ca^{2+}	0.37	39.6	107
intact factor XIa, 0.5 mM Ca^{2+}	1.11	18.4	16.58
intact factor XIa, 0 Ca^{2+}	10.0	9.9	0.99
factor XIa light chain, 5 mM Ca^{2+}	8.93	7.98	0.89
factor XIa light chain, 0 Ca^{2+}	10.0	6.0	0.60

Comparison of the Enzymatic Activities of Factor XIa and Its Light Chain against the Natural Substrate Factor IX. Optimal rates for the activation of factor IX by factor XIa require the presence of calcium ions. We have presented evidence in our previous report (Sinha et al., 1985) that the heavy chain of factor XIa has a major role in the Ca^{2+}-dependent activation of factor IX by factor XIa. It is also clear from the results presented in Figure 1 that a heavy chain specific antibody inhibits the enzyme only in the presence of Ca^{2+} and does not do so in its absence. The initial rates of activation of the two enzymes as obtained from Figure 1 are summarized in Table I. The activities of the two enzymes against the natural substrate pyro-Glu-Pro-Arg-pNA are also shown for comparison. In order to determine whether the loss of activity of the isolated light chain of factor XIa or that of intact factor XIa in the absence of calcium is due to a lack of interaction of factor IX with the heavy chain of factor XIa, we studied the kinetics of the reaction using both intact factor XIa and also its isolated light chain as the enzyme both in the presence and in the absence of Ca^{2+}, and the results are shown in Figure 2. In the activation of factor IX by factor XIa, linear double-reciprocal plots were obtained in the absence or in the presence of 5.0 or 0.5 mM Ca^{2+}. However, at Ca^{2+} ion concentrations of 0.10 and 0.25 mM Ca^{2+}, nonlinear double-reciprocal plots were obtained probably reflecting heterogeneous catalysis, and therefore, these data are not presented. The kinetic parameters derived from these experiments are also summarized in Table II. First, it is apparent that the kinetic parameters obtained with the isolated light chain in the presence or absence of Ca^{2+} and those obtained with intact factor XIa in the absence of Ca^{2+} are similar, although the V_{max} obtained with the isolated light chain is slightly lower in the absence than in the presence of Ca^{2+}. The most im-

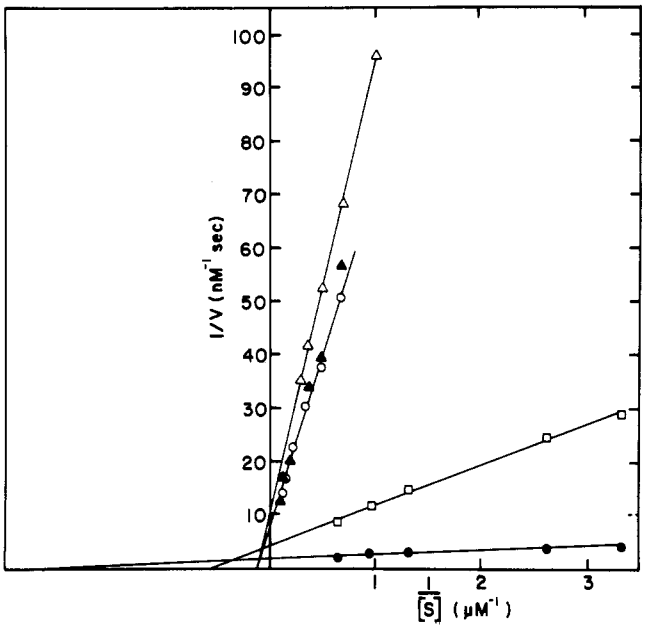

FIGURE 2: Comparison of the enzymatic activity of factor XIa (1.0 nM) or its light chain (1.0 nM) against factor IX in the presence and absence of calcium. Details of the assay are described in the text. Results shown are activation by factor XIa (●) or by the light chain of factor XIa (▲) in the presence of 5 mM Ca^{2+}, activation by factor XIa (□) in the presence of 0.5 mM Ca^{2+}, and activation by factor XIa (○) or by the light chain of factor XIa (△) in the presence of 2 mM EDTA.

portant point derived from this experiment is that the absence of Ca^{2+} and the absence of the heavy chain of factor XIa have similar effects on the kinetics of factor IX activation, i.e., a major (approximately 30-fold) increase in K_m with a lesser (4–6-fold) decrease in k_{cat}.

Kinetics of Activation of Factor IX by Factor XIa in the Presence of the Light Chain Specific Antibody 5F4. The Lineweaver–Burk plots of the activation of factor IX by factor XIa in the presence of various concentrations of the anti-factor XI antibody 5F4 are shown in Figure 3. It is clear from the plots that the binding of 5F4 to factor XIa does not affect the K_m of the reaction but alters its V_{max}. In other words, the catalytic site is blocked, reducing the effective enzyme concentration, while the enzyme–substrate binding site remains unaffected. This is an example of classical noncompetitive inhibition.

Kinetics of Activation of Factor IX by Factor XIa in the Presence of the Heavy Chain Specific Antibody 3C1. The Lineweaver–Burk plots of the activation of factor IX by factor XIa in the presence of the various concentrations of the heavy chain specific anti-factor XI antibody 3C1 are shown in Figure 4. The double-reciprocal plots yielded patterns consistent with classical competitive inhibition; i.e., the V_{max} of the reaction is unaffected by the antibody whereas progressively higher concentrations of the antibody yielded progressively higher values of K_m. Thus, the binding of 3C1 to factor XIa reduces the effective concentration of the binding sites of the enzyme for its substrate factor IX while the catalytic site remains unaltered.

Calculation from RIA Data of Binding Constants for Antigen–Antibody Complex Formation. From eq 1, one needs to know the total concentration of the antibody and the concentration of the antibody bound in order to determine the binding constant. A plot of $[\text{antibody}]_{bound}/[\text{antibody}]_{free}$ vs. $[\text{antibody}]_{bound}$ should give a straight line, from the slope of which the binding constant of the antigen–antibody complex can be determined. The Scatchard plots of the binding of the

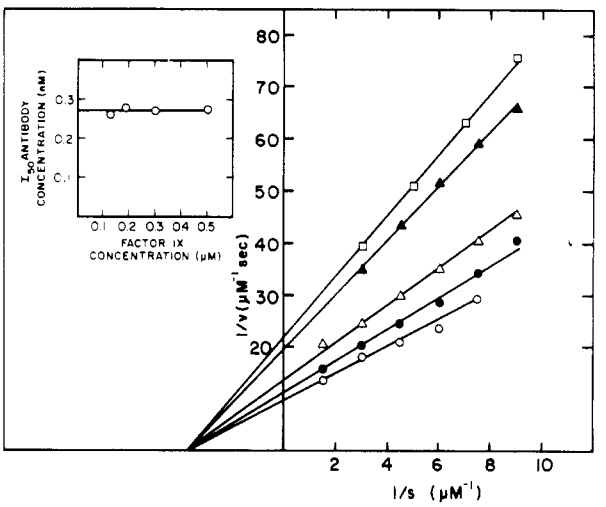

FIGURE 3: Double-reciprocal plots of the activation of factor IX by factor XIa in the presence of various concentrations of the light chain specific antibody 5F4. Factor XIa was incubated with various concentrations of the antibody for 20 min at 37 °C and added to the assay buffer (50 mM Tris, 100 mM NaCl, and 5 mg/mL BSA, pH 7.5), made 5 mM in $CaCl_2$ and containing various concentrations of a mixture of 9 parts of unlabeled factor IX and 1 part of ^3H-labeled factor IX. Final concentration of factor XIa in the assay mixture was 0.23 nM. Factor IX was used in the range of 0.11–0.66 μM. At various time points, aliquots were withdrawn and assayed with TCA-soluble peptide as described under Experimental Procedures. Initial velocities were calculated from points where less than 10% of the substrate was cleaved. Results shown are the double-reciprocal plots in the absence (○) and in the presence of 0.1 (●), 0.166 (△), 0.27 (▲), or 0.30 nM (□) 5F4. The inset is the plot of I_{50} obtained by the procedure of Cha et al. (1975) against various substrate concentrations.

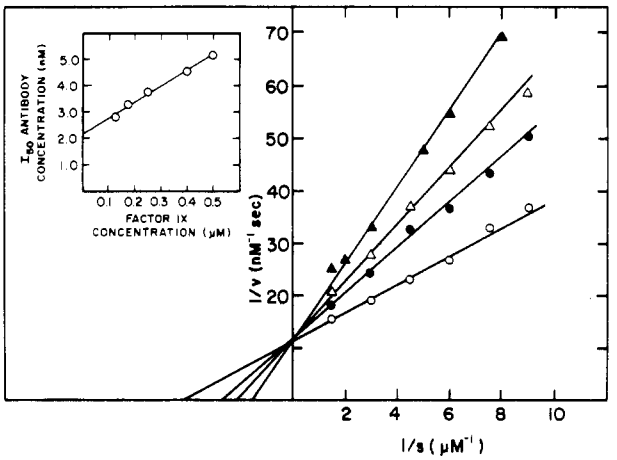

FIGURE 4: Double-reciprocal plots of the activation of factor IX by factor XIa in the presence of various concentrations of the heavy chain specific antibody 3C1. The experimental procedures were the same as described under Figure 3. The concentrations of the antibody in the plots are 0.0 (○), 1.33 (●), 2.66 (△), and 4.0 nM 3C1 (▲). The inset is the plot showing the relationship of I_{50} values to the substrate concentrations.

antibody 5F4 to factor XI and to factor XIa are shown in Figure 5, and those for 3C1 are shown in Figure 6. From the slopes, the value of K for the binding of antibody 5F4 to factor XI is 0.99×10^{10} (Figure 5A) and to factor XIa, 0.78×10^{10} (Figure 5B). Comparable binding constants for antibody 3C1 are 0.34×10^{10} for factor XI (Figure 6A) and 0.20×10^{10} for factor XIa (Figure 6B).

Comparison of Binding Constants for Antigen–Antibody Complex Formation Obtained from RIA Data and from Kinetic Parameters. The inhibitor constant K_i (the dissociation constant of the enzyme–inhibitor complex) both for a com-

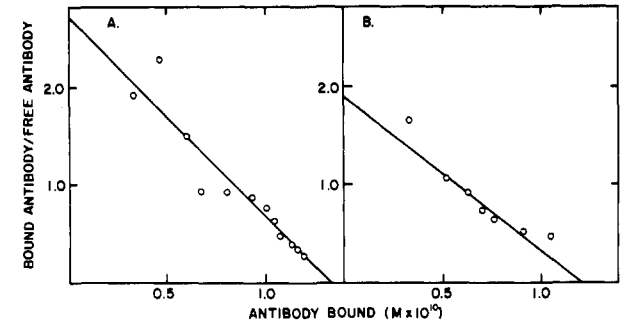

FIGURE 5: Binding of the light chain specific antibody 5F4 to factor XI (A) and factor XIa (B). RIA binding data are plotted in modified Scatchard form as described in the text. From the slopes of the plots, the dissociation constant of the antibody when factor XI was used as the antigen was 1.01×10^{-10} M whereas it was 1.28×10^{-10} M when factor XIa was the antigen.

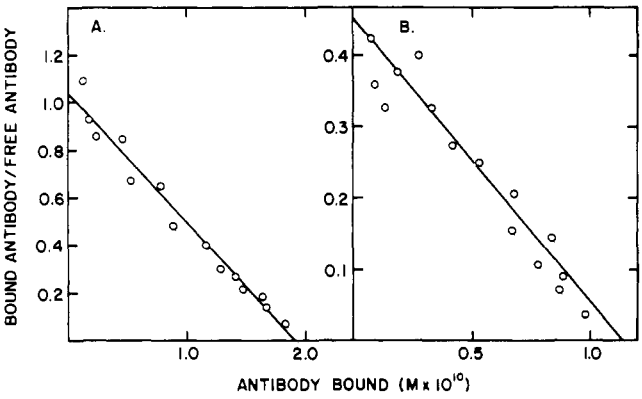

FIGURE 6: Binding of the heavy chain specific antibody 3C1 to factor XI (A) and factor XIa (B). RIA binding data are plotted in modified Scatchard forms. From the slopes of the plots, the dissociation constants of the antibody were 2.9×10^{-10} and 5.0×10^{-10} M for factor XI and factor XIa, respectively.

petitive and also for a noncompetitive inhibitor can be determined from the double-reciprocal plots under conditions where the equilibrium concentration of the inhibitor can be replaced by its total concentration (Segal, 1974b). This is true only when the binding is not very tight. In the present case, the inhibitor constant is the dissociation constant of the antigen–antibody complex. The binding constants of both the antibodies to factor XIa used in the present study are relatively tight as determined by the RIA method. Since the classical methods of analyzing the kinetic data for determining the inhibitor constant are inadequate for tight binding inhibitors, we used the method of I_{50} developed by Cha (1975) for the evaluation of the inhibitor constants. I_{50} is the total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction. In the case of competitive inhibition, I_{50} is related to the substrate concentration according to eq 2. It can be easily seen from this equation that a plot of I_{50} vs. substrate concentration should be a straight line from the slope of which K_i can be determined. The inset of Figure 4 represents such a plot when the antibody 3C1 was used as the inhibitor. The calculated value of K_i from the slope (2.0×10^{-9} M) is in fairly good agreement with the value ($K_d = 0.5 \times 10^{-9}$ M) obtained when the solid-phase RIA method was used.

In the case of noncompetitive inhibition, I_{50} is independent of the substrate concentration and related to the enzyme concentration according to eq 3. Thus, K_2 can be evaluated by determining the values of I_{50} at different enzyme concentrations. However, since we were mainly interested in the role of the heavy chain of factor XIa, we studied the kinetics in the presence of the light chain specific antibody 5F4 using only one concentration of the enzyme. Nonetheless, the inset of Figure 3 shows that I_{50} is independent of substrate concentration as it should be. Also, from the value of I_{50} and total enzyme concentration, K_i is calculated to be 2.1×10^{-10} M, which is in very good agreement with the value of 1.28×10^{-10} M determined by the RIA method.

Discussion

Although there are certain general features exhibited by all enzymatic reactions, each enzyme seems to be unique with respect to the relationships between its structural domains and its functional properties. Enzyme-catalyzed reactions show a distinctive feature not usually observed in nonenyzmatic reactions, namely, the phenomenon of saturation with substrate. The saturation effect led to a general theory of enzyme action and kinetics. According to this theory, which is basic to the quantitative analysis of all aspects of enzyme kinetics and inhibition, the enzyme first reacts with the substrate to form an enzyme–substrate complex, which then breaks down in a second step to form free enzyme and the product(s). Thus, the theory assumes that the enzyme, in addition to possessing a catalytic center, has a binding site(s) for the substrate. Only when the substrate is anchored on this site can it undergo the structural change that converts it to the product. The study of enzyme inhibition is a useful tool to explore the physical and chemical architecture of the active site and the kinetic mechanism of the reaction. Factor XIa is the only enzyme in the coagulation cascade that consists of four polypeptide chains held together by disulfide bonds and has two active sites each associated with each of the two identical light chains. Factor XIa is the activator of factor IX in the intrinsic pathway of blood coagulation. In this reaction, the presence of both calcium ions and also the heavy chain of factor XIa is essential for optimal rates of factor IX activation even though the active site is contained entirely within the light chain. The mechanisms by which calcium ions and the heavy chain of factor XIa promote the reaction have been unclear. The present project was undertaken to explore this mechanism by studying the calcium-dependent and calcium-independent activation of factor IX both by factor XIa and by its isolated light chain in the presence of two monoclonal antibodies directed against two different epitopes of factor XIa.

When the activation of factor IX was examined by using either the light chain of factor XIa (Figure 1C,D) or the intact factor XIa as the enzyme (Figure 1A,B), the light chain specific antibody (5F4) inhibited the activation rate almost completely both in the presence and in the absence of Ca^{2+}. The inhibitory effect by the light chain specific antibody (5F4) on the activation of factor IX either by intact factor XIa or by its light chain fragment both in the presence and in the absence of calcium is not unexpected in view of the fact that the entire enzymatic active site of factor XIa is associated with its light chain.

The heavy chain specific antibody (3C1) did not have any effect on activation rates either in the presence or in the absence of Ca^{2+} only when the factor XIa light chain was used as the enzyme (Figure 1C,D). In contrast, when intact factor XIa was used as the enzyme (Figure 1A,B), the same antibody (3C1) inhibited the activation rate (>80%) in the presence of Ca^{2+} whereas in the absence of Ca^{2+}, it showed a slightly stimulatory effect. The slight stimulatory effect of the heavy chain specific antibody (3C1) on the capacity of intact factor XIa to activate factor IX in the absence of Ca^{2+} may be explained by a change in conformation of the molecule when the antibody binds to its heavy chain, thereby rendering it

enzymatically more effective. We have also shown that the same heavy chain specific antibody, 3C1, enhances the activation of factor XI by factor XIIa, a reaction in which Ca^{2+} is not required (Akiyama et al., 1985).

To determine the cause of the reduced activity of intact factor XIa due to the absence of calcium or of the isolated light chain of factor XIa in the presence or absence of calcium, we compared intact factor XIa and the isolated light chain for their activities toward a small synthetic substrate as well as the natural substrate factor IX both in the presence and in the absence of Ca^{2+}. Lineweaver–Burk plots of the two enzymes against the synthetic substrate (data not shown) clearly indicate that they are almost indistinguishable. On the other hand, when the activation of the natural substrate factor IX was studied, the two enzymes behaved quite differently (Figure 2). Factor XIa was much more potent in the presence of Ca^{2+} than in its absence. On the contrary, the presence or absence of Ca^{2+} made very little difference in the case of the isolated light chain of factor XIa. Moreover, the enzymatic activity of the light chain was almost identical with that of intact factor XIa when Ca^{2+} was absent.

The activation of factor IX (FIX) by factor XIa (FXIa) in the absence or in the presence of Ca^{2+} can be described as follows:

$$\begin{array}{l} \text{FXIa} + \text{FIX} \rightleftharpoons \text{FXIa-FIX} \xrightarrow{k_p} \text{FXIa} + \text{FIXa} \\ + \\ \text{FIX-Ca}^{2+} \\ \Downarrow \\ \text{FXIa-FIX-Ca}^{2+} \xrightarrow{\beta k_p} \text{FXIa} + \text{FIXa-Ca}^{2+} \end{array}$$

From the double-reciprocal plots (Figure 2) of the activation of factor IX by factor XIa in the absence of Ca^{2+}, the K_m and k_{cat} (k_p) of the reaction are 10 μM and 9.9 min^{-1}, respectively. In the presence of an optimal concentration (5 mM) of Ca^{2+}, the value of K_m is lowered to 0.37 μM whereas the value of k_{cat} (βk_p) is increased to 39.6 min^{-1}. Thus, the combined effects of K_m and k_{cat} will increase the rate of activation by more than 100-fold in the presence of an optimal concentration of calcium. When the initial rate of activation of factor IX by factor XIa was calculated from Figure 1, it was found to be approximately 2 orders of magnitude more rapid in the presence of Ca^{2+} than in its absence (Table I). Moreover, the light chain either in the presence or in the absence of calcium has catalytic activity equivalent to intact factor XIa in the absence of calcium. Thus, the decrease in activity of intact factor XIa due to the absence of calcium is similar to that observed when its active-site-containing light chain is separated from its heavy chain. In order to explore further the relationships between the functional properties and physical domains of factor XIa, we have analyzed the kinetic parameters for the activation of factor IX by factor XIa in the presence of antibodies specific to different epitopes within the enzyme.

The Lineweaver–Burk plots of the activation of factor IX by factor XIa in the presence of various concentrations of the light chain specific antibody 5F4 (Figure 3) exhibited patterns typical of classical noncompetitive inhibition. In other words, the K_m values for the inhibited and uninhibited reactions are the same, whereas lower values of V_{max} are obtained with higher concentrations of the antibody. Thus, the binding of 5F4 to factor XIa reduces the effective concentration of the enzyme. Knowing that the light chain of factor XIa possesses the catalytic site, the observed noncompetitive inhibition by 5F4 reported here is not unexpected. Although we have not studied the kinetics using different enzyme concentrations to evaluate the value of the inhibitor constant (in this case the dissociation constant of the antigen–antibody complex), we have shown that the value of I_{50} is independent of the substrate concentration (inset, Figure 3) as expected for an example of noncompetitive inhibition (Cha, 1975). Although evaluated from one concentration of the enzyme, the value of the inhibitor constant is in very good agreement with that obtained from the RIA method.

One interesting observation reported in this paper is derived from the kinetic data obtained with the heavy chain specific antibody 3C1. The Lineweaver–Burk plots in the presence of various concentrations of 3C1 showed patterns typical of classical competitive inhibition; i.e., the binding of 3C1 to the enzyme does not alter the V_{max} of the reaction, whereas higher values of K_m are obtained with higher concentrations of the antibody. Thus, the inhibitor (3C1) interferes with the binding of the substrate to the enzyme. The inhibitor constant obtained from the plot of I_{50} vs. substrate concentration (inset, Figure 4) is 2.0×10^{-9} M, in fairly good agreement with the value of K_d (0.5×10^{-9} M) determined by the RIA method.

The noncompetitive inhibition by the light chain specific antibody 5F4 does not provide any new information other than a confirmation of previously published reports that the active site of the molecule is associated with its light chain. However, the inhibition of the activation by the heavy chain specific antibody 3C1 can be explained in several ways: (1) the antibody binds to a site on the heavy chain which is very close to the active site and thus inhibits the reaction by steric hindrance; (2) although 3C1 binds to the heavy chain, the conformation of the molecule around the active site on the light chain is altered when the antibody binds to the enzyme, such that a putative substrate binding site on the light chain is obscured with a resultant increase in K_m; (3) the conformation of the enzyme is such that an epitope on its heavy chain recognized by 3C1 actually represents the enzyme–substrate binding site (or at least a part of it). If the 3C1 binding site is very close to the active site and as a result the active site is partially or totally blocked, then the binding of 3C1 to factor XIa should have resulted in noncompetitive inhibition; however, purely competitive inhibition is observed (Figure 4). Therefore, inhibition by mechanism 1 is ruled out. We have no direct evidence to disprove mechanism 2. However, this possibility seems remote on the basis of additional evidence concerning the functional properties of factor XIa and its isolated light chain and concerning the kinetic effects of calcium ions on factor IX activation by factor XIa. First, the predominant effect of removal of the heavy chain (i.e., when the isolated light chain is the catalyst) is a major increase in K_m (Figure 2, Table II), i.e., similar to the effect of antibody 3C1. Second, the predominant effect of the absence of calcium ions is a major increase in the K_m of factor IX activation by intact factor XIa. Third, the absence of calcium ions has no significant effect upon factor IX activation by the isolated light chain of factor XIa. Fourth, the competitive inhibition by antibody 3C1 of factor IX activation by intact factor XIa occurs only in the presence of calcium ions; in the absence of calcium ions, no inhibition is observed. These observations mitigate strongly against the possibility of mechanism 2 and in favor of mechanism 3, which is depicted schematically in Figure 7 for the monomer of factor XIa. Inhibition by antibody 5F4 is also shown for comparison. According to the model, the binding of calcium ions to factor IX induces a conformational alteration (Bajaj, 1982) which allows the molecule to bind to an enzyme–substrate binding site on the heavy chain of factor IX, thereby leading to optimal rates of

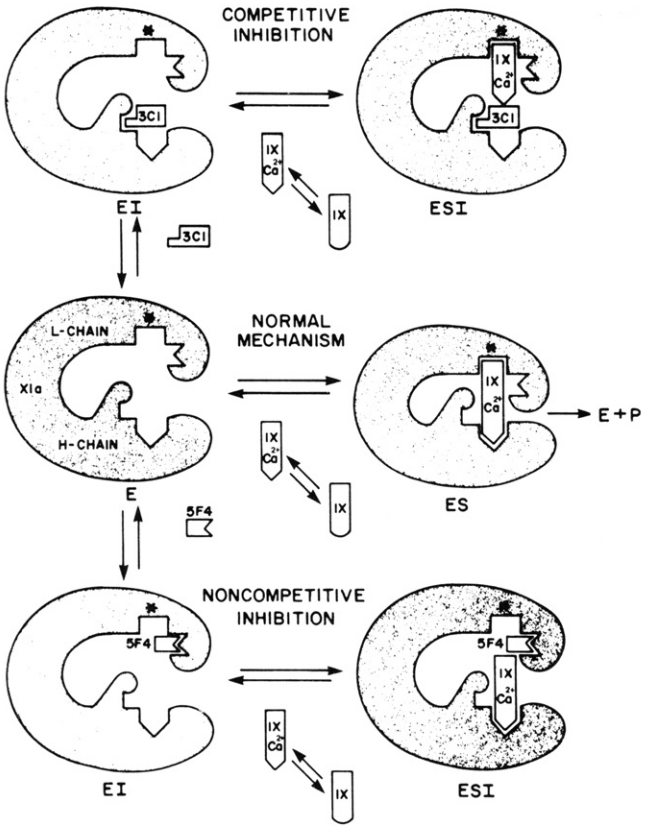

FIGURE 7: Schematic diagram of the mechanism of factor XIa catalyzed factor IX activation and effects of monoclonal antibodies. The mechanism is discussed in the text. The schematic diagram was made for the monomer of factor XIa.

catalysis. This hypothesis explains (1) the competitive inhibition in the presence of antibody 3C1, (2) the noncompetitive inhibition by antibody 5F4, (3) the equivalent activity of factor XIa and the isolated light chain in the absence of calcium ions, (4) the kinetic effects of calcium ions on factor IX activation by factor XIa, and (5) the fact that in the presence of calcium ions the light chain of factor XIa (fully enzymatically active against a small peptide substrate) is 2 orders of magnitude less active as a factor IX activator than intact factor XIa. A definitive test of this hypothesis will require direct binding studies of factor IX to the heavy chain of factor XIa.

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Registry No. Factor IX, 9001-28-9; factor IXa, 37316-87-3; factor XIa, 37203-61-5; Ca, 7440-70-2.

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