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Kinetic Comparison of Bovine Blood Coagulation Factors IXaα and IXaβ toward Bovine Factor X†

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ABSTRACT: The V_{max}/K_m (μM^{-1} min^{-1}) for bovine factor X activation by bovine factor IXaα, in the presence of sufficient [Ca^{2+}] to saturate the initial reaction rate, was 0.007. When factor IXaβ was substituted for factor IXaα in this reaction, the V_{max}/K_m decreased to 0.001, suggesting that factor IXaα was a more potent catalyst under these conditions. When phospholipid (PL) vesicles (egg phosphatidylcholine/bovine brain phosphatidylserine, 4:1 w/w) were added to these same systems, at levels sufficient to saturate their effects, little change in the V_{max}/K_m occurred when factor IXaα was the enzyme. However, when factor IXaβ was employed, the V_{max}/K_m dramatically increased to 0.023, demonstrating that factor IXaβ responded to PL addition to a much greater extent than did factor IXaα. Upon addition of thrombin-activated factor VIII (factor VIIIa,t), at a suboptimal level, to the above systems, the V_{max}/K_m for factor X activation by factor IXaα/Ca^{2+}/PL/factor VIIIa,t was increased to 1.0, whereas this parameter for factor X activation by factor IXaβ/Ca^{2+}/PL/factor VIIIa,t under the same conditions was found to be 27.3. During these studies, it was discovered that the factor X which became activated to factor Xa during the course of reaction participated in several feedback reactions: activation of factor X, activation of factor VIII, and conversion of factor IXaα to factor IXaβ. All feedback reactions, which are capable of complicating the kinetic interpretation, were inhibited by performing the studies in a system which contained a rapid factor Xa inhibitor, Glu-Gly-Arg-CH_2Cl, thus allowing kinetic constants to be accurately determined. The results show that while factor IXaα is a more efficient enzyme than factor IXaβ toward factor X activation in the absence of cofactors, the response of factor IXaβ to the reaction cofactors, PL and factor VIIIa,t, is much greater than that of factor IXaα.

Factor IXa is an enzyme which possesses proteolytic, amidolytic, esterolytic, and thioesterolytic activity. This enzyme exists in the plasma in an inactive form, factor IX, which is activated through a series of proteolytic events. One activator of factor IX is the protease factor XIa in the presence of Ca^{2+} (Schiffman et al., 1963; Kingdon et al., 1964). Here, two steps are involved (Fujikawa et al., 1974). First factor XIa catalyzes cleavage of an Arg_{146}–Ala_{147} peptide bond in single chain (M_r 55 400) factor IX, yielding factor IXα. Next, the Arg_{181}–Val_{182} bond is cleaved, liberating an activation glycopeptide of molecular weight of approximately 10 000 and yielding a two-chain enzyme, factor IXaβ, of M_r ~44 400. Factor IX is also activated by a protease (RVV-X),[1] present in the venom

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[1] Abbreviations: RVV-X, the coagulant protein from the venom of Russell's viper; $NaDodSO_4$, sodium dodecyl sulfate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tris, tris(hydroxymethyl)aminomethane; PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid.

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of Russell's viper, by a variation of the above mechanisms (Lindquist et al., 1978). In this case, only the Arg_{181}–Val_{182} peptide bond is cleaved, resulting in a two-chain, disulfide-linked enzyme, factor IXaα, of approximate M_r 55 400.

The role of factor IXa in intrinsic blood coagulation is to catalyze conversion of the plasma zymogen factor X to factor Xa. Bovine factor X is a two-chain glycoprotein, of M_r ~54 000. Activation of this zymogen occurs as a consequence of cleavage of an Arg_{51}–Ile_{52} peptide bond and concomitant release of a glycopeptide in the active site containing heavy chain (Fujikawa et al., 1972, 1975). In the intrinsic coagulation system, factor X activating activity has been proposed to be optimally present in a mixture of factor IXa/Ca^{2+}/phospholipid (PL)/factor VIII (Chuang et al., 1972). Here, it is believed that the enzyme responsible for factor X activation is factor IXa (Suomela & Blomback, 1977; Hultin & Nemerson, 1978) and that factor VIII, its potency greatly enhanced after activation to factor VIIIa by thrombin (Hultin & Nemerson, 1978), is a cofactor for this reaction. It is envisioned that factor X activation occurs by this complex in which factor IXa and factor VIIIa are bound to a PL matrix and stabilized thereon by Ca^{2+} (Chuang et al., 1972).

Since two forms of factor IXa can exist, we decided to evaluate their catalytic efficiency in the factor X activation system. We felt that since it was possible and economical for the organism to provide factor IXa by a single peptide bond cleavage (factor IXaα), there may be a rationale, involving the activity of this enzyme, for the intrinsic coagulation system to generate a form of factor IXa (factor IXaβ) in which two peptide bonds were cleaved. This paper presents a comparison of these two forms of factor IXa as enzymes in factor X activation.

Materials and Methods

Proteins. Bovine factor IX was purified from fresh citrated bovine plasma by the procedure of Fujikawa et al. (1973), as modified by Amphlett et al. (1979). Bovine factor IXaα was prepared from factor IX after activation by Sepharose 4B–RVV-X, as described by Byrne & Castellino (1978). Bovine factor IXaβ was prepared by activation of factor IX with factor XIa, as described by Amphlett et al. (1979). The concentration of each enzyme was determined by titration with NPGB (Byrne et al., 1980).

For activation of bovine factor IX with factor XIa, a crude preparation of factor XIa was employed, which was effective in this regard. The starting material for this preparation was the barium citrate supernatant used for the factor IX preparation. Celite 501 (40 g/L) was added to the supernatant and stirred for 1 h at 4 °C. Factor XI adsorbs to Celite and becomes activated during the process. The Celite was then washed in a funnel with a solution of 1% NaCl, until the absorbance at 280 nm was negligible. Factor XIa was eluted from the Celite by mixing with a solution of 10% NaCl for 1 h at 4 °C The eluate was subjected to Millipore filtration to remove fine particles of Celite and concentrated by ultrafiltration. The crude factor XIa was stored in 50% glycerol/10 mM benzamidine hydrochloride at −20 °C. Prior to use, the factor XIa was assayed by using a slight operational modification of the method of Kurachi & Davie (1977).

The RVV-X used to activate factor IX to factor IXaα was purified as described by Amphlett et al. (1982). Here, the QAE-Sephadex pool of venom obtained from the Miami Serpentarium was employed (Amphlett et al., 1982). RVV-X was insolubilized to Sepharose 4B according to the method described by Byrne & Castellino (1978).

Bovine factor X_1 was purified by modification of the procedure of Fujikawa et al. (1972), as described by Bajaj et al. (1977), and tritiated in its sialic acid residues by the procedure of van Lenten & Ashwell (1971). Our exact procedure for preparation of $AcNeu^7$-[^{3}H]factor X, of specific activity of approximately 240 000 dpm/μg, has been described (Amphlett et al., 1982). When assayed by the coagulant assay of Bachmann et al. (1958), the radiolabeled factor X possessed a specific activity virtually identical with that of native factor X.

Bovine factor Xa was prepared by activation of factor X with insolubilized RVV-X. Factor X (0.5 mg/mL) in 0.025 M Tris-HCl/0.1 M NaCl, pH 7.4, was added to Sepharose 4B–RVV-X, equilibrated in the same buffer, at a ratio of 8:1 (v/v) factor X:resin. The activation mixture was adjusted to 5 mM in $CaCl_2$ and gently mixed at room temperature. Full factor Xa activity, assayed with NPGB, was usually observed after 15 min. The activation mixture was then passed over a small column containing Chelex-100 resin, and the resin was washed with a small amount of buffer. The active site concentration of factor Xa was determined with NPGB and the purity evaluated by $NaDodSO_4$/polyacrylamide gel electrophoresis. Factor Xa was divided into appropriate aliquots and stored at −20 °C.

Bovine factor VIII was isolated from fresh, citrated bovine plasma employing modifications of the methods of Vehar & Davie (1980) and Legaz & Davie (1976). In particular, bovine blood was collected fresh at a local slaughterhouse in 10-L plastic buckets containing 1 L of anticoagulant solution. The anticoagulant consisted of 28.5 g of trisodium citrate/8 g of benzamidine/50 mg of soybean trypsin inhibitor/25 mg of heparin/1.7 mg of Trasylol (FBA Pharmaceuticals) per L of solution. Plastic ware was used in all subsequent steps. The plasma was separated from the cells by centrifugation at 9000 rpm in a fixed-angle GS-3 rotor for 30 min at 4 °C. To 5.3 L of plasma, obtained from 10 L of blood, was added 1 mL of 1 M DFP, and the plasma was stirred for 20 min. In the cold room, the plasma was brought to 20% saturation with ultrapure ammonium sulfate, by slow addition of 605 g of the salt to a gently stirring solution. The suspension was stirred for an additional 40 min and then subjected to centrifugation for 10 min at 8000 rpm in a GS-3 rotor at 4 °C. The supernatant was discarded. The precipitate was brought to room temperature and dissolved in 250 mL of 20 mM Tris-HCl/200 mM NaCl/10 mM benzamidine, pH 6.8. The solution was centrifuged for 10 min, at 8000 rpm in a GS-3 rotor at 15 °C, to remove any undissolved material. The solution was then adjusted to 15 °C and the temperature maintained with a water bath, while glycine (15.75 g/100 mL) was slowly added until the solution was 2.1 M in glycine. After an additional 5 min, the suspension was centrifuged for 10 min at 6000 rpm in a GS-3 rotor at 15 °C. The supernatant was discarded. The precipitate was brought to room temperature and cut into small pieces. The precipitate was then gently stirred with 300 mL of 50 mM Tris-HCl/200 mM NaCl/1 mM benzamidine, pH 7.4 (column buffer), containing 0.3 mL of stock Trasylol, until it redissolved. The solution was then centrifuged for 10 min at 6000 rpm to remove any undissolved material in a GS-3 rotor at 20 °C. This solution was then divided into 25-mL aliquots and stored at −20 °C.

A 25-mL sample of redissolved glycine precipitate was fractionated by chromatography on CL-Sepharose-4B at room temperature. If the sample was fresh, the absorbance at 280 nm was adjusted to 45 units/mL with the addition of column buffer. If the sample was frozen, it was thawed in a 37 °C

water bath and insoluble material removed, prior to adjusting the concentration. A maximum of 30 mL of sample was applied to a CL-Sepharose 4B column (4 cm × 90 cm), equilibrated in column buffer. The flow rate was adjusted to 15 mL/h, and 3.8-mL fractions were collected. The eluate from the column was monitored by measurement of absorbance at 280 nm. The protein fractions containing factor VIII activity were identified by a specific clotting assay. The fractions containing maximum factor VIII activity were pooled and chilled. The factor VIII pool was concentrated by placing the solution in a dialysis bag (M_r 10000 cutoff) and laying the bag in a bed of dry PEG-20000 at 4 °C, until the desired volume was obtained. The concentrated factor VIII pool was stored in aliquots at −75 °C. Reduced $NaDodSO_4$/polyacrylamide gel electrophoresis of the sample revealed the presence of a single band of approximate M_r 200000. The specific activity of the preparation was 30 units/mg, prior to thrombin activation.

Thrombin was isolated from activation of purified bovine prothrombin by a factor Xa/factor Va/Ca^{2+}/phospholipid system. A typical activation mixture (5 mL) contained 60 mg of prothrombin/1 mg of factor Xa/3000 units of factor Va (Sigma Chemical Co.)/0.24 μmol of phospholipid (rabbit brain cephalin)/200 μmol of Ca^{2+}. The activation mixture was incubated at room temperature until maximum thrombin activity was observed by a thrombin coagulation assay. This activation was then terminated by the addition of benzamidine to a final concentration of 5 mM. Thrombin was isolated from the activation mixture, as described by Lundblad et al. (1976). The activation mixture was dialyzed against 0.025 M sodium phosphate, pH 6.5, and then applied to a sulfopropyl-Sephadex (C-50) column (2.5 cm × 18 cm), equilibrated in the same buffer. The column was washed with the equilibrating buffer and factor Xa eluted in this fraction. A 0.1 M sodium phosphate, pH 6.5, buffer was then used to further wash the column until the absorbance at 280 nm was minimal. Thrombin was then eluted from the column with 0.25 M sodium phosphate, pH 6.5. The thrombin fraction was then concentrated by ultrafiltration and stored at −75 °C in usable aliquots.

Preparation of Synthetic Phospholipid (PL) Vesicles. Egg phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) were purchased from the Sigma Chemical Co. and further purified by preparative thin-layer chromatography. For the preparation of synthetic PL vesicles, an amount of PC and PS necessary to obtain a 4:1 (w/w) ratio was mixed in $CHCl_3/CH_3OH$ (95:5 v/v), and evaporated to dryness under a stream of N_2. The sample was then suspended in a solution of 0.15 M NaCl, pH 7.0, and subjected to sonication for 30 min with a Heat Systems Model 200 R probe sonicator. The resulting suspension was centrifuged for 90 min at 120000*g* in a Beckman Model L2-65B ultracentrifuge. The supernatant was used as the source of the small PL vesicles. The phospholipid concentration was determined by phosphate analysis (Lowry & Lopez, 1946).

Assay for Enhancement of Factor VIII Coagulant Activity. The enhancement of factor VIII coagulant activity by either thrombin or factor Xa was monitored by the specific factor VIII coagulation assay (Legaz & Davie, 1976). For the thrombin stimulation of factor VIII, a reaction mixture consisting of the desired concentrations of factor VIII (0.5–5 units/mL) and thrombin (0.01–0.1 unit/mL) in 25 mM Tris-HCl/100 mM NaCl, pH 7.4, was incubated in a 37 °C water bath. At selected time intervals, an aliquot of the incubation mixture was diluted and assayed for factor VIII activity.

The factor Xa stimulation of factor VIII activity was performed in a similar manner. The reaction mixture consisted of the desired amount of factor VIII (0.5–5 units/mL) and factor Xa (0.25 μg/mL) in 25 mM Tris-HCl/100 mM NaCl/5 mM $CaCl_2$/40 μM PL, pH 7.4. The reaction was carried out at 37 °C, and aliquots were assayed at selected time intervals.

The amount of thrombin or factor Xa in the assay mixture was controlled such that it would not contribute to the clotting time of factor VIII coagulation assay.

Factor X Activation Assay. For all experiments described, the conversion of factor X to factor Xa was monitored by the peptide release assay developed by Silverberg et al. (1977). All assays were performed in polypropylene tubes in a 37 °C water bath. The assay buffer was 25 mM Tris-HCl/100 mM NaCl, pH 7.4. Protein solutions were prepared with the assay buffer, except factor VIII which was in assay buffer with 1 mM benzamidine hydrochloride.

In a particular example, 1 μg/mL factor IXa was incubated with 40 μM PL and 8.3 mM $CaCl_2$. The reaction was initiated with the addition of the desired amount of $AcNeu^7$-[^{3}H]factor X in a final volume of 0.3 mL. At specific time intervals, a 0.04-mL aliquot of the activation mixture was added to 0.04 mL of a solution consisting of 0.03 M EDTA/3 mg/mL bovine serum albumin, pH 7.4 at 4 °C, in order to arrest the activation. After this, 0.04 mL of cold 15% trichloroacetic acid was added to each sample, and the suspension was centrifuged for 3 min in an Eppendorf 5413 centrifuge. An 0.08-mL sample of the supernatant was added to 0.32 mL of H_2O in a 7-mL scintillation vial. A 4-mL volume of scintillation cocktail was added to the vial, and the contents were thoroughly mixed. The amount of activation peptide present was determined in a Beckman LS-100 liquid scintillation counter. A similar experiment, performed in the absence of factor IXa, was used to determine the fraction of factor X which was soluble in Cl_3CCOOH. This value, which represented less than 2% of the precipitable tritium, was subtracted from experimental points where necessary. The level of radioactivity expected for 100% activation of factor X was determined from the maximum radioactivity obtained when factor X was cleaved by RVV-X. The data were converted to picomoles per milliliter factor X activated and plotted against time. The earliest linear portion of this line was used to determine the initial rate. Values for K_m and V_{max} were determined from Lineweaver–Burk plots.

When factor VIII was included in the activation mixture, factor VIII, factor IXa, PL, and $CaCl_2$ were preincubated at 37 °C for 3 min, before the activation was initiated with factor X (the order of addition of components in the preincubation mixture did not affect the results). When thrombin-activated factor VIII was employed in the assay, factor VIII was preincubated with thrombin for 2 min, followed by addition of PL, Ca^{2+}, and factor IXa, and the mixture was incubated for an additional 1 min before the activation was initiated with the addition of factor X. The amount of thrombin and length of preincubation were determined for maximal factor VIIIa activity by using factor VIII clotting assays.

Results

The physical, chemical, and enzymic characteristics of all proteins used in this study have been described in the indicated previous publications from this laboratory. The lone exception to this is factor VIII. The final factor VIII isolated by the

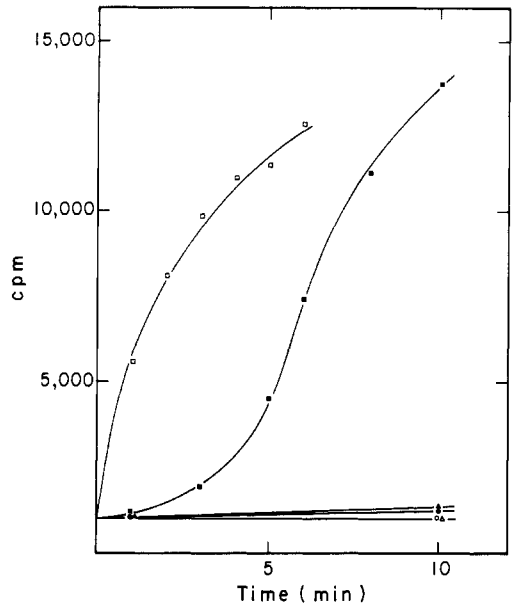

FIGURE 1: Activation of AcNeu[7]-[^{3}H]factor X by factor IXaα. The factor IXaα concentration was 4.1 μg/mL, in 25 mM Tris-HCl/100 mM NaCl, pH 7.4 at 37 °C, (○) in the absence of Ca^{2+}, (●) in the presence of 8 mM Ca^{2+}, and (▲) in the presence of 8 mM Ca^{2+} and 87 μM PL. The factor IXaα concentration was 2.3 μg/mL, (■) in the presence of 8 mM Ca^{2+}, 87 μM PC/PS, and 0.5 unit/mL factor VIII and (□) as above, with thrombin- (0.04 unit/mL) activated factor VIII. (△) Factor IXaα was absent from the activation mixture.

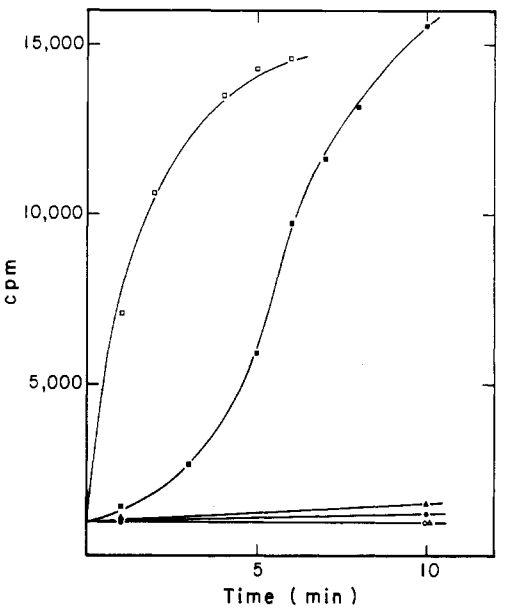

FIGURE 2: Activation of AcNeu[7]-[^{3}H]factor X by factor IXaβ. The factor IXaβ concentration was 4.1 μg/mL, in 25 mM Tris-HCl/100 mM NaCl, pH 7.4 at 37 °C, (○) in the absence of Ca^{2+}, (●) in the presence of 8 mM Ca^{2+}, and (▲) in the presence of 8 mM Ca^{2+} and 87 μM PL. The factor IXaβ concentration was 2.1 μg/mL, (■) in the presence of 8 mM Ca^{2+}, 87 μM PC/PS, and 0.5 unit/mL factor VIII and (□) as above, with thrombin- (0.04 unit/mL) activated factor VIII. (△) Factor IXaβ was absent from the activation mixture.

purification method described herein possessed a similar specific activity to that described by Legaz & Davie (1976), although our final yield was approximately half of that obtained in the previous purification method. Most importantly, however, the factor VIII used in this study was activated by 30–100-fold by thrombin. The final degree of activation was dependent upon the concentration of factor VIII and thrombin employed. Thus, this material was entirely suitable for the studies described herein.

Examples of factor X activation by factors IXaα and IXaβ, in the presence of various combinations of accessory components, as assayed by the peptide release method, are shown in Figures 1 and 2, respectively. The overall patterns of activation observed with the addition of the cofactors were similar for both forms of factor IXa. When factor IXa was omitted from the reaction mixture, no detectable level of factor Xa was formed, under all combinations of conditions. For the concentration ranges employed, no activation was detected in the absence of Ca^{2+}. A slow, measurable rate of activation was produced by factor IXa and Ca^{2+}. This rate was stimulated when PL was added to the activation mixture. A major increase in the rate of activation was observed when factor VIII was added to the system. This increase in factor Xa formation was preceded by a lag phase. Preactivation of factor VIII with thrombin eliminated the lag phase and further increased the reaction rate. The disappearance of the lag phase upon addition of activated factor VIII implies that unactivated factor VIII became activated during the reaction and that the activated form of factor VIII is the effective cofactor. The apparent stimulation of factor VIII appears to coincide with the formation of low levels of factor Xa.

As can be seen from Figures 1 and 2, the general response to cofactors appeared the same for factors IXaα and IXaβ. However, there were notable differences in the rate of factor Xa formation by the two forms of factor IXa. In the presence of only Ca^{2+}, factor IXaα appears to be more effective toward factor X activation than factor IXaβ. Although both forms

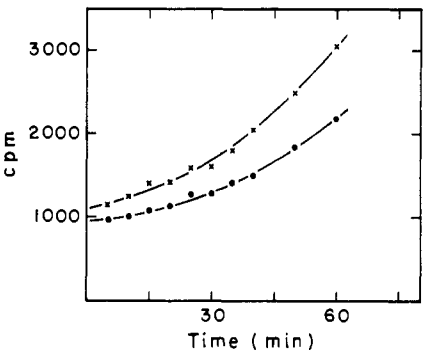

FIGURE 3: Time course of the activation of AcNeu[7]-[^{3}H]factor X by factor IXaβ, Ca^{2+}, and PL. The factor IXaβ concentration was 1 μg/mL, with 8 mM Ca^{2+} and 40 μM PC/PS in 25 mM Tris-HCl/100 mM NaCl, pH 7.4 at 37 °C; (●) the factor X concentration was 75 μg/mL; (×) the factor X concentration was 90 μg/mL.

of factor IXa appeared to have rates which are stimulated by the addition of PL, the stimulation was more extensive for factor IXaβ. In the presence of both Ca^{2+} and PL, a faster rate of factor X activation was obtained with factor IXaβ than with factor IXaα. While these conclusions are apparent from examination of Figures 1 and 2, it should be emphasized that they were drawn from experiments designed to optimally calculate this effect (see below). The data presented in Figures 1 and 2 do clearly show, however, that factor IXaβ continued to produce faster rates of factor Xa formation, when factor VIII/VIIIa was added to the system. Therefore, differences were observed with factors IXaα and IXaβ in terms of their ability to cleave factor X. Furthermore, although factor IXaβ appeared to be a more efficient enzyme in the presence of Ca^{2+} and PL and in the presence of Ca^{2+}, PL, and factor VIII/VIIIa, factor IXaα appeared to be more effective in the presence of Ca^{2+} alone.

The rates of factor Xa activation by factor IXa were very slow when factor VIII and/or factor VIIIa was not present in the system. When data at longer times were collected for

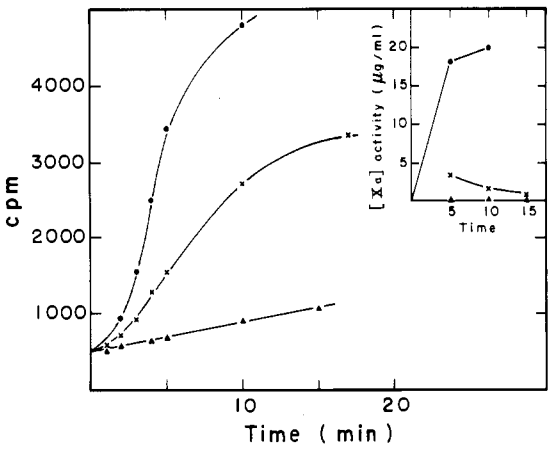

FIGURE 4: Activation of factor X by factor IXaβ, Ca^{2+}, PL, and factor VIII in the absence and presence of GGA. AcNeu^7-[^{3}H]factor X, at 47 μg/mL, was incubated with 1.7 μg/mL factor IXa, 0.3 unit/mL factor VIII, and 40 μM PC/PS in 25 mM Tris-HCl/100 mM NaCl/9 mM $CaCl_2$, pH 7.4 at 37 °C, (●) in the absence of GGA, (×) in the presence of 5×10^{-6} M GGA, and (▲) in the presence of 5×10^{-5} M GGA. (Inset) The amount of active factor Xa was determined with S-2222 for the above mixtures.

activation of factor X by factor IXa in the presence of Ca^{2+} and PL, the rate of factor Xa formation did not appear linear. Figure 3 demonstrates this effect, showing the results obtained for two factor X concentrations by factor IXaβ with Ca^{2+} and PL. Since active factor Xa is the product of the reaction, it appears as though factor Xa contributes to the observed rate of factor X activation by factor IXa in the presence of Ca^{2+} and PL. The kinetic parameters of this particular feedback effect have been previously published (Link & Castellino, 1982).

Before a detailed study of the activation of factor X by factors IXaα and IXaβ could be performed, the possibility that factor Xa might influence the reaction kinetics through other feedback reactions required investigation. From analysis of the kinetic parameters previously obtained for activation of factor X by factor Xa (Link & Castellino, 1982), it does appear as though this reaction exerts considerable influence on the activation of factor X in the absence of factor VIII/VIIIa. Further, as seen in Figures 1 and 2, it is likely that factor Xa is responsible for stimulation of the activation rate of factor X by activation of factor VIII, when unactivated factor VIII is used in the initial activation mixture (Hultin & Nemerson, 1978). Finally, for a valid comparison between factors IXaα and IXaβ, it must be determined whether factor Xa is capable of converting factor IXaα to IXaβ.

In order to link more closely the enhancement in factor VIII activity to the formation of factor Xa, a rapid and irreversible inhibitor of factor Xa, Glu-Gly-Arg-CH_2Cl (GGA), generously donated by Dr. Elliot Shaw, was added to the assays of AcNeu^7-[^{3}H]factor X activation by factor IXa/Ca^{2+}/PL/factor VIII. GGA did not inhibit factor IXaα or factor IXaβ toward synthetic substrates at the concentrations used herein. Therefore, only the product, factor Xa, should be inhibited by the concentrations of GGA employed in the factor X activation assays.

Figure 4 illustrates the effect of two different concentrations of GGA on activation of AcNeu^7-[^{3}H]factor X in the factor IXaβ/Ca^{2+}/PL/factor VIII system. In the absence of GGA, a lag phase, followed by a rapid rate of factor Xa production, was observed. When 5 μM GGA was included in the same activation mixture, approximately a 70% decrease in the rate of factor Xa formation was noted, following the lag phase. An additional 10-fold increase in the GGA concentration further

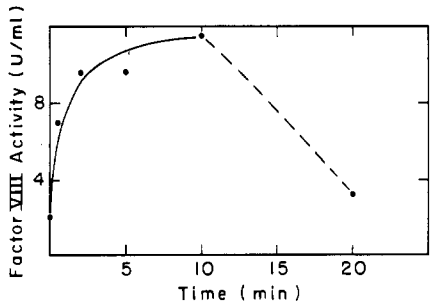

FIGURE 5: Activation of factor VIII by factor Xa. Factor VIII (1.4 units/mL) was incubated with 0.26 μg/mL factor Xa in 50 mM Tris-HCl/250 mM NaCl/5 mM $CaCl_2$, pH 8.3, with 40 μM PC/PS at 37 °C. The factor VIII activity was determined with specific factor VIII coagulation assays.

reduced the rate of factor Xa production in this system to approximately 5% of the rate found in the absence of GGA. The amount of active factor Xa present in the assay was determined with the synthetic chromogenic substrate, *N*-BzO-Ile-Glu-Gly-Arg-pNA (S-2222). The insert of Figure 4 shows that active factor Xa was available for feedback activation of factor VIIIa at 5 and 10 min with 5 μM GGA and that no measurable active factor Xa was detected at 50 μM GGA. Therefore, it appears as though factor Xa does exhibit feedback activation in the factor IXa/Ca^{2+}/PL/factor VIII activation system, enhancing the factor VIII activity, which results in an increase in the rate of factor Xa formation. Evidence of factor Xa enhancement of factor VIII activity was also obtained from factor VIII coagulation assays. Figure 5 illustrates this effect. Under the conditions described, aliquots were removed at various times, diluted 1200-fold, and assayed for factor VIII clotting activity. Control samples, containing all components other than factor VIII, did not decrease the clotting time of factor VIII deficient plasma. From Figure 5, it can be seen that factor VIII activity increased 8–10-fold at 5–10 min, followed by a decrease in factor VIII optimal activity. Routinely, a 5–10-fold enhancement in factor VIII was obtained with 0.15–0.3 μg/mL factor Xa. Perhaps greater stimulatory activity is possible at higher levels of factor Xa, but this could not be tested since these increased concentrations of factor Xa would interfere with the factor VIII coagulant assay. Similar results have been previously obtained for activation of bovine (Vehar & Davie, 1980) and human (Griffith et al., 1982) factor VIII by factor Xa. Factor IXaα and factor IXaβ did not activate bovine factor VIII.

It has been shown that factor Xa can activate factor IX to factor IXaβ (Kalousek et al., 1975). Therefore, the conversion of factor IXaα to IXaβ by factor Xa seemed very probable. The conversion was monitored by nonreduced 5% NaDodSO$_4$/polyacrylamide gel electrophoresis. The conversion of factor IXaα to factor IXaβ by factor Xa/Ca^{2+}/PL was conducted at 37 °C. The gel patterns, seen in Figure 6, show that factor IXaα is converted to factor IXaβ by factor Xa in this system. In the absence of PL (data not shown) the conversion occurs much more slowly. A control experiment with factor IXaα, with Ca^{2+} and PL at 37 °C, showed no increase in the amount of factor IXaβ with time, up to 60 min. When GGA, at 2.5×10^{-5} M, was added to the factor IXaα mixture with factor Xa/Ca^{2+}, in the presence or absence of PL, significant inhibition of conversion of factor IXaα to IXaβ is seen from the gel patterns of Figure 6. Therefore, it appears that factor Xa could convert factor IXaα to IXaβ and that the level of conversion could be minimized with GGA.

Since the factor Xa formed in activation of factor X exhibited reaction feedback characteristics on the substrates

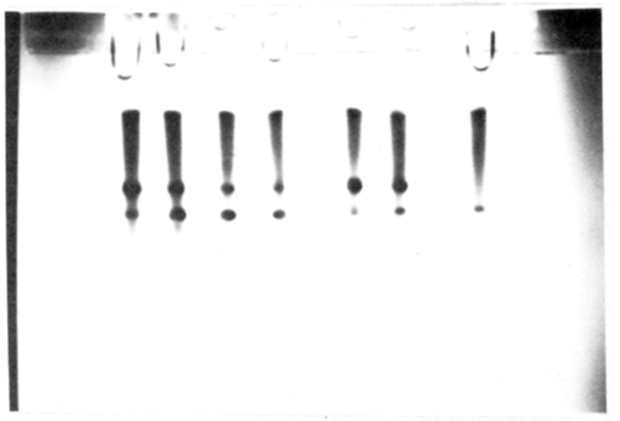

FIGURE 6: Conversion of factor IXaα to factor IXaβ by factor Xa in the absence and presence of GGA was monitored by 5% Na-DodSO$_4$/polyacrylamide gel electrophoresis. Factor IXaα (0.29 mg/mL) in 50 mM Tris-HCl/100 mM NaCl/8 mM $CaCl_2$ was incubated with 8.5 μg/mL factor Xa for times of 10, 20, 40, and 60 min (tubes 1–4, respectively) and in the presence of 2.5×10^{-5} M GGA for 60 min (tube 6). Tube 5 is the initial factor IXaα, and tube 7 is factor IXaβ.

factor X, factor VIII, and factor IXaα, any useful evaluation of the kinetics of various activation systems should be designed such that these reactions were inhibited. As was seen, this can be accomplished with the use of GGA as a rapid and specific factor Xa inhibitor in the factor IXa activation system. Therefore, all activation mixtures contained sufficient GGA (2.5×10^{-5} M) to inhibit the factor Xa produced.

The initial rates of factor X activation, by factor IXaα, as a function of the initial concentrations of factor X, in the presence of Ca^{2+} and GGA, were determined and subjected to analysis by Lineweaver–Burk plots (data not shown). The initial rate of factor Xa formation was proportional to the factor IXaα concentration between 1 and 10 μg/mL active enzyme. The particular experiments were conducted at a factor IXaα concentration of 4.1 μg/mL. For each point obtained for this experiment, and all other experiments reported, it was determined that the factor Xa concentration was a linear function of time over the period of assay, ensuring that initial rates of factor Xa formation were indeed being monitored. The plot obtained was linear, yielding a V_{max} of 0.11 $\pm$ 0.01 pmol of Xa min^{-1} (pmol of IXaα)$^{-1}$ and a $K_{m,app}$ of 16.2 $\pm$ 2.0 μM factor X. Similar data for factor X activation by factor IXaβ were obtained, under identical conditions as for activation by factor IXaα. In this case, it was found that the initial rate of factor Xa production was proportional to the factor IXaβ concentration over a range of 4.0–40 μg/mL factor IXaβ. A factor IXaβ concentration of 19.4 μg/mL was employed for the kinetic study, from which a V_{max} of 0.019 $\pm$ 0.03 pmol of Xa min^{-1} (pmol of IXaα)$^{-1}$ and a $K_{m,app}$ of 16.0 $\pm$ 2.0 μM were calculated from Lineweaver–Burk analysis of the data.

Lineweaver–Burk plots for the activation of AcNeu^7-[^{3}H]factor X by factor IXaα and IXaβ, in the presence of Ca^{2+}, PL, and GGA, were also obtained. The rate of factor Xa formation was proportional to the level of factor IXaα and factor IXaβ present for a concentration range of 2–10 μg/mL. The PC/PS mixture employed produced optimal effects for both enzymes, between 20 and 90 μM. Therefore, an enzyme concentration of 6.0 μg/mL and a PC/PS concentration of 40 μM was employed in the experiments. From these data, a V_{max} of 0.062 $\pm$ 0.005 pmol of Xa min^{-1} (pmol of IXaα)$^{-1}$ and a $K_{m,app}$ of 9.1 $\pm$ 0.5 μM factor X were calculated for factor IXaα. Similarly, a V_{max} of 0.052 $\pm$ 0.005 pmol of Xa min^{-1} (pmol of IXaβ)$^{-1}$ and a $K_{m,app}$ of 2.2 $\pm$ 0.04 μM factor X were obtained for factor IXaβ.

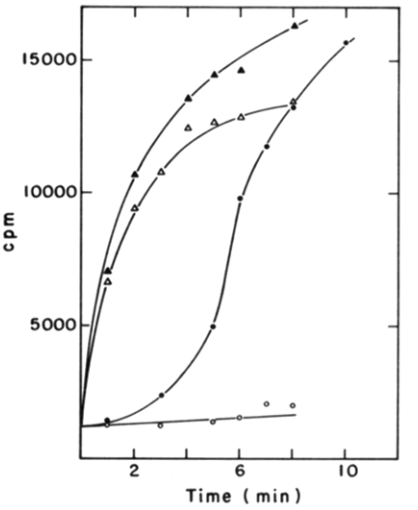

FIGURE 7: Activation of AcNeu^7-[^{3}H]factor X by factor IXaβ with PC/PS, Ca^{2+}, and factor VIII/VIIIa in the absence and presence of GGA. Factor X at 47 μg/mL was activated with 2.1 μg/mL factor IXaβ in 25 mM Tris-HCl/100 mM NaCl/8 mM $CaCl_2$, pH 7.4 at 37 °C, containing (●) 0.5 unit/mL factor VIII, (○) 0.5 unit/mL factor VIII, with GGA, (▲) 0.5 unit/mL factor VIII, activated with 0.04 unit/mL thrombin, and (△) 0.5 unit/mL factor VIII, activated with 0.04 unit/mL thrombin, with GGA. The GGA concentration was 2.5×10^{-5} M, when present; the PC/PS concentration was 40 μM.

The effect of factor VIII/VIIIa on the activation of factor X with factor IXaβ and the effect of GGA on these activations are seen in Figure 7. The inhibition of factor Xa by GGA eliminated the rate enhancement seen for factor VIII, but not that seen with thrombin-activated factor VIIIa. Thus, it is clear that the lag period noted with factor VIII, in great part, is due to the activation of factor VIII by factor Xa produced during the activation. Similar results were seen with factor IXaα. In order to compare the effect of factor VIII on factor X activation by each factor IXa, factor VIIIa was employed, at subsaturating concentrations, with saturating levels of Ca^{2+} and PC/PS, and GGA. Under the above conditions the rate of factor Xa formation was proportional to factor IXaα between 0.2 and 0.8 μg/mL. When the factor X concentration was varied, 0.37 μg/mL factor IXaα was employed. The data obtained were plotted according to Lineweaver–Burk treatment. Linear graphs were obtained, yielding a V_{max} of 2.54 $\pm$ 0.4 pmol of Xa min^{-1} (pmol of IXaα)$^{-1}$ and a $K_{m,app}$ of 2.5 $\pm$ 0.3 μM factor X for the factor IXaα/Ca^{2+}/PL/factor VIIIa system. Lower concentrations of factor IXaβ were required in this system. The rate of factor Xa formation was proportional to 0.02–0.06 μg/mL factor IXaβ. An activation mixture consisting of 0.038 μg/mL factor IXaβ, with Ca^{2+}, PC/PS, and factor VIIIa, was used in experiments in which the factor X was varied. The data obtained were treated according to a Lineweaver–Burk plot. A linear graph was obtained, yielding a V_{max} of 13.4 $\pm$ 1.0 pmol of Xa min^{-1} (pmol of IXaβ)$^{-1}$ and a $K_{m,app}$ of 0.49 $\pm$ 0.06 μM factor X.

The initial rate data for factor X activation by factors IXaα and IXaβ were obtained at five to seven factor X levels at concentrations from 0.01 to 2 $\times$ the K_m for the reactions. It was found that when higher concentrations of factor X were employed, feedback reactions with rapidly generated factor Xa were more difficult to control.

All kinetic parameters obtained were based on at least two separate determinations at each substrate concentration. The factor IXaβ preparations did not contain factor IXaα, when evaluated by 5% NaDodSO$_4$/polyacrylamide gel electrophoresis. However, small amounts (<10%) of factor IXaβ were

Table I: Kinetic Parameters of Factor X Activation by Factor IXaα and IXaβ

composition of factor X activating mixture[a]	$K_{m,app}$ (μM)	V_{max} [pmol of Xa min^{-1} (pmol of IXa)$^{-1}$]	V_{max}/K_m (μM^{-1} min^{-1})
factor IXaα, Ca^{2+}	16.2 ± 2.0	0.11 ± 0.01	0.007
factor IXaα, Ca^{2+}, PL (40 μM)	9.1 ± 0.05	0.062 ± 0.005	0.007
factor IXaα, Ca^{2+}, PL (40 μM), factor VIIIa (0.5 unit/mL)	2.5 ± 0.3	2.54 ± 0.4	1.0
factor IXaβ, Ca^{2+}	16.0 ± 2.0	0.019 ± 0.003	0.001
factor IXaβ, Ca^{2+}, PL (40 μM)	2.2 ± 0.04	0.052 ± 0.005	0.023
factor IXaβ, Ca^{2+}, PL (40 μM), factor VIIIa (0.5 unit/mL)	0.49 ± 0.06	13.4 ± 1.0	27.3

[a] The Ca^{2+} concentration was 8 mM in all the activation mixtures, and GGA, at 2.5×10^{-5} M, was also present in the activation mixture.

present in factor IXaα preparations, which could not be readily removed. While this latter contamination would not influence the interpretation of any results obtained in the absence of factor VIII, a correction for this level of factor IXaβ would be desirable in the kinetics performed in the presence of factor VIIIa, since approximately 10-fold greater concentrations of factor IXaα, compared to that of factor IXaβ, were used in these experiments. This correction was readily accomplished. In order to do so, it was first shown that the rates obtained with the stock factor IXaα were the sum of the contributions of factors IXaα and IXaβ. This was accomplished by first accurately determining the concentration of factor IXaβ in the factor IXaα sample by scanning the stained $NaDodSO_4$ gels, at a variety of different concentrations of samples and standard preparations. Next, a series of experiments were performed in which factor IXaβ was added to constant and variable levels of factor IXaα and, similarly, in which factor IXaα (together with its small known levels of factor IXaβ) was added to varied and constant levels of factor IXaβ, in the presence of factor VIIIa, and analyzed in the complete activation system. Upon analysis of all data, it was found that when the contribution of factor IXaβ to the rate, determined from experiments with only factor IXaβ present, was subtracted from the total rate, the resulting rate of factor Xa formation was proportional to the amount of factor IXaα in the sample. Thus, the rates of factor X activation by factor IXaα were corrected for the presence of the known amounts of factor IXaβ present therein, by employing the $K_{m,app}$ and V_{max} values obtained for factor IXaβ, under the same conditions. These corrections were only of importance in the complete activation system, viz., factor X, factor IXa, Ca^{2+}, PL, and factor VIIIa, and were applied to data thus obtained. The same corrections were found to be trivial in all activation systems without added factor VIIIa.

A summary of the kinetic constants for all activation mixtures is presented in Table I. Since the factor VIIIa levels are subsaturating in our systems, the data of Table I would be best used for comparative purposes.

Discussion

Factor IXa, in complex with factor VIII/VIIIa, PL, and Ca^{2+}, activates factor X to factor Xa in the intrinsic coagulation pathway. To date, two different forms of factor IXa have been isolated. One form, factor IXaα, is generated from factor IX by cleavage of a single peptide bond, whereas another factor IXaβ is produced by cleavage of two peptide bonds in factor IX and concomitant release of an activation peptide. In an attempt to understand the rationale for production of a complex form of factor IXa, when a simple molecule of appropriate activity can be generated, we have undertaken a study in which the activity of the two forms of the enzyme, and their respective responses to addition of cofactors, was measured toward its physiologic substrate, factor X. In addition, this comparative study would allow us to evaluate structure–function relationships of the protease, factor IXa. In order to perform this study in a rigorous manner, an understanding of the possible feedback reactions of factor Xa on components of the activation system was required. Also, a stable and activatable sample of factor VIII was necessary. The preparation of factor VIII, isolated as described, fulfilled these requirements, yielding an activation response as high as that obtained in any previously reported study, while showing the characteristic patterns of factor VIII on $NaDodSO_4$/polyacrylamide gel electrophoresis (Schmer et al., 1972; Legaz et al., 1973).

There appeared to be three feedback reactions of factor Xa that could interfere with the assays for the activation of factor X by factor IXa. These are the activation of factor X by factor Xa, the activation of factor VIII by factor Xa, and the conversion of factor IXaα to IXaβ by factor Xa. It was important to understand and to be able to control these reactions. In this paper, the activation of factor VIII by factor Xa and the conversion of factor IXaα to IXaβ were studied for this purpose. The activation of factor X by factor Xa was explored in greater detail in a previous publication from this laboratory (Link & Castellino, 1982). We found that a K_m of 0.06 μM and a V_{max} of 1.7 min^{-1} characterized the factor X activation by factor Xa at pH 7.4 and 37 °C, at saturating Ca^{2+}. PC/PS had a slight inhibitory effect, chiefly due to an increase in the K_m of the reaction.

An assessment of the importance of the factor Xa pathway can be made by comparison of the kinetic parameters of the factor Xa activation of factor X to those published for components of either the extrinsic or intrinsic pathways. The $K_{m,app}$ and V_{max} determined for activation by the factor IXa/factor VIIIa/PL/Ca^{2+} complex were 0.063 μM and 500 mol of factor Xa min^{-1} (mol of factor IXa)$^{-1}$, respectively (van Dieijen et al., 1981). The factor VIIa/Ca^{2+}/tissue factor complex produced a k_{cat} of 32 s^{-1} and a K_m of 0.34 μM (Silverberg et al., 1977). In the absence of factor VIIIa, the V_{max} for the factor IXa/Ca^{2+}/PL system decreased approximately 20000-fold, and in the absence of tissue factor the k_{cat} for the factor VIIa/Ca^{2+} activation system decreased 2900-fold and the K_m increased 10-fold. Therefore, factor Xa activation of factor X could significantly contribute to the activation rate of factor X in both activation systems, in the absence of the cofactors, factor VIIIa and tissue factor.

The activation of factor VIII by factor Xa, by product feedback reaction during factor X activation assays, was suggested in an earlier study (Hultin & Nemerson, 1978). The increased rate of factor Xa formation that follows the lag phase in factor X activation assays with factors IXa and VIII, PL, and Ca^{2+} indicated that factor VIII was being activated in the system. The ability of a factor Xa inhibitor to decrease or eliminate the effects of activated factor VIII in this assay implies that factor VIII is activated by a feedback reaction of factor Xa. Hultin (1982), using GGA at concentrations that did not totally inhibit factor Xa, observed a prolonged lag phase and decreased initial rate in factor X activations by factor IXa/VIII/PL/Ca^{2+}. Furthermore, it was found that a specific thrombin inhibitor did not prolong the lag phase

(Hultin, 1982). More direct evidence for the activation of factor VIII by factor Xa is seen in the enhanced factor VIII coagulant activity, when factor VIII is preincubated with factor Xa, PL, and Ca^{2+} (Figure 5). There does appear to be a requirement for both PL and Ca^{2+} for this activation. Factor Xa has been shown to require Ca^{2+} for reactions with factors IX (Kalousek et al., 1975), VII (Radcliffe & Nemerson, 1975), and X (Jesty et al., 1974) and prothrombin (Nesheim et al., 1979), while PL has been shown to enhance the activations of factor IX (Kalousek et al., 1975), factor VII (Radcliffe & Nemerson, 1975), and prothrombin (Nesheim et al., 1979; Rosing et al., 1980).

The conversion of factor IXaα to factor IXaβ was important if a comparative study was to be performed of factors IXaα and IXaβ activation of factor X. A study of the conversion was performed with Ca^{2+} in the absence and presence of PL and in the absence and presence of GGA. The NaDodSO$_4$/polyacrylamide gel analysis showed that the conversion of factor IXaα to IXaβ proceeded at a much faster rate in the presence of PL. This is in agreement with the report that PL and Ca^{2+} were required for the activation of factor IX by factor Xa (Kalousek et al., 1975). Furthermore, it was found that the conversion could be inhibited with GGA.

A complex composed of factors IXa and VIIIa, PL, and Ca^{2+} is considered to serve as activator in the intrinsic activation of factor X. There is strong evidence that factor IXa is the enzyme in the factor X activating complex and that factor VIIIa, PL, and Ca^{2+} act as cofactors to factor IXa. In the presence of PL and Ca^{2+}, factor IXa was able to activate factor X (Hultin & Nemerson, 1978). While factor VIIIa was shown to enhance the activation, it was unable to activate factor X without factor IXa (Hultin & Nemerson, 1978). Recently, factor IXaβ, alone, was shown to activate factor X at a very slow rate that could be greatly increased with the addition of Ca^{2+}, PL, and factor VIIIa (van Dieijen et al., 1981). In that publication, kinetic parameters for different factor X activation mixtures were determined, and it was found that both PL and factor VIIIa caused important changes of the kinetic parameters of factor X activation by factor IXaβ. The presence of PL resulted in a decrease in the K_m, while the addition of factor VIIIa to the factor IXa/PL/Ca^{2+} complex led to increases in the V_{max}.

The present study was concerned with the differences in factors IXaα and IXaβ and the influence of the cofactors on these two forms of factor IXa in regard to activation of factor X. Factor IXaβ is believed to be the physiological form of factor IXa and was used in most previous studies. An evaluation of the influence of the cofactors of factor IXaβ in the activation of factor X suggests interesting cofactor effects. A minimum complex composition of factor IXaβ and Ca^{2+} was required for an observable rate of factor Xa formation. In the presence of various combinations of the factor Xase components, no detectable level of factor X activation occurred if either factor IXa or Ca^{2+} was omitted from the system. When PL was employed with factor IXaβ and Ca^{2+}, there was an increased in the initial rate of factor Xa formation. The kinetic parameters determined for factor X activation by factor IXaβ and Ca^{2+}, in the absence and presence of PL, indicate that the accelerated rate in the presence of PL is primarily due to a decrease in the $K_{m,app}$, although there was also an observed increase in the V_{max}. The activations conducted in the presence of factors IXa/VIIIa/PL/Ca^{2+} were greatly enhanced as compared to activations in the absence of the factor VIIIa cofactor. The primary effect of factor VIIIa on the activation of factor X, seen in the kinetic parameters, is a 40–260-fold increase in the V_{max}, depending on the factor IXa used. The kinetic parameters for the activation of factor X by factor IXaβ with the various cofactors differ from those reported by van Dieijen et al. (1981). This is likely due to major differences in experimental conditions. The present study employed different enzyme and substrate concentrations, a different PL system, and lower factor VIIIa concentrations. However, the general effects of PL and factor VIII on the kinetic parameters of factor X activation were the same in both studies.

Thus, there are definite differences in the activity of factors IXaα and IXaβ toward factor X and in factor IXaα and IXaβ interactions with their cofactors. Since the behavior of the two forms of factor IXa is very similar in regard to synthetic substrates and inhibitors (Link & Castellino, 1983), it would appear that the structural differences in the two factor IXa forms do not affect the active site region. The interaction of factor IXa with its physiological substrate, factor X, and with its cofactors, PL and factor VIIIa, is more likely to be influenced by regions of the enzyme which are distant from the active site than factor IXa interactions with small substrates and inhibitors. The ability of factor IXaβ to interact with PL more effectively than factor IXaα may indicate that a region of factor IXa which can bind PL is more readily exposed in factor IXaβ than in factor IXaα. The region of factor IXa interaction with factor VIIIa may also be more available in factor IXaβ. The effect of factor VIIIa on factor IXaβ was much greater than that on factor IXaα, but it is difficult to evaluate whether this effect is due to direct action of factor VIIIa on the enzyme or whether it effects the interaction of the enzyme with PL and Ca^{2+}.

Finally, it should be pointed out that abnormal factor IX molecules exist such as factor IX Chapel Hill, which are activated only to factor IXaα (Briet et al., 1982). On the basis of the findings reported in the present paper, it would be expected that the factor X activation ability of this abnormal activated factor IX would be defective in systems requiring all components of the factor X activation system, as has been found (Briet et al., 1982). We conclude that this abnormal activation could occur due to the fact that the enzyme formed is factor IXaα. Obviously, other abnormalities in this defective molecule could contribute to its decreased activity, but until its entire sequence is known, no further conclusions can be forwarded.

Registry No. Ca, 7440-70-2; blood coagulation factor IXaα, 66526-17-8; blood coagulation factor IXaβ, 66526-18-9; blood coagulation factor X, 9001-29-0; blood coagulation factor Xa, 9002-05-5; blood coagulation factor VIIIa, 72175-66-7.

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Kinetics of Bovine Milk Lipoprotein Lipase and the Mechanism of Enzyme Activation by Apolipoprotein C-II†

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ABSTRACT: The kinetics of bovine milk lipoprotein lipase (LPL) were studied in order to determine the reaction mechanism of this enzyme. Reaction velocities were determined at varying concentrations of emulsified trioleoylglycerol (TG) and different fixed concentrations of apolipoprotein C-II (C-II) or at varying C-II concentrations and different fixed concentrations of TG. Neither the apparent K_m(TG) nor the apparent K_m(C-II) was affected by varying the concentrations of C-II or TG, respectively. However, C-II increased the apparent V_{max} for the enzyme about 20-fold. The following kinetic parameters were calculated from Lineweaver–Burk plots: K_m(C-II) = 2.5×10^{-8} M and K_m(TG) = 2.5×10^{-3} M. The dissociation constant (K_S) of the enzyme–TG binary complex was determined from Scatchard plots to be 7.6×10^{-8} M. Heparin was found to be a competitive dead-end inhibitor against both TG and C-II. Tricapryloylglycerol represented a competitive inhibitor against TG but a noncompetitive inhibitor against C-II. C-II was shown to interact with dansylated bovine milk LPL, increasing its fluorescent emission by inducing a conformational change in the enzyme. Based on these studies, it was concluded that the LPL-catalyzed reaction follows a random, bireactant, rapid-equilibrium mechanism and the role of C-II in the activation process involves an increase in the catalytic rate constant (k_p) resulting from conformational changes of LPL induced by C-II.

Lipoprotein lipase (LPL,[1] EC 3.1.1.34) is an enzyme which catalyzes the hydrolysis of the triacylglycerols of chylomicrons and very low density lipoproteins (VLDL), thus playing a key role in the metabolism of the plasma lipoproteins. It is well established that LPL is an enzyme which acts at endothelial cell surfaces of various tissues and can be liberated into the

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[1] Abbreviations: LPL, lipoprotein lipase; C-II, apolipoprotein C-II; TG, trioleoylglycerol; FA, fatty acid(s); S_p, pseudosubstrate; DNS-LPL, dansylated lipoprotein lipase; VLDL, very low density lipoprotein(s); DNS-Cl, 5-(dimethylamino)-1-naphthalenesulfonyl chloride; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane.

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