Characterization of the Clotting Activities of Structurally Different Forms of Activated Factor IX

Enzymatic Properties of Normal Human Factor IXaα, Factor IXaβ, and Activated Factor $IX_{Chapel\ Hill}$

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Abstract

Two structurally different forms of activated human Factor IX (Factor IXaα and IXaβ) have been previously reported to have essentially identical clotting activity in vitro. Although it has been shown that activated Factor $IX_{Chapel\ Hill}$, an abnormal Factor IX isolated from the plasma of a patient with mild hemophilia B, and normal Factor IXaα are structurally very similar, the clotting activity of activated Factor $IX_{Chapel\ Hill}$ is much lower (approximately fivefold) than that of normal Factor IXaβ. In the present study we have prepared activated Factor IX by incubating human Factor IX with calcium and Russell's viper venom covalently bound to agarose. Fractionation of the activated Factor IX by high-performance liquid chromatography demonstrated the presence of both Factors IXaα and IXaβ. On the basis of active site concentration, determined by titration with antithrombin III, the clotting activities of activated Factor $IX_{Chapel\ Hill}$ and IXaα were similar, but both activities were <20% of the clotting activity of Factor IXaβ. Activated Factor IX activity was also measured in the absence of calcium, phospholipid, and Factor VIII, by determination of the rate of Factor X activation in the presence of polylysine. In the presence of polylysine, the rates of Factor X activation by activated Factor $IX_{Chapel\ Hill}$, Factor IXaα, and Factor IXaβ were essentially identical. We conclude that the clotting activity of activated Factor $IX_{Chapel\ Hill}$ is reduced when compared with that of Factor IXaβ but essentially normal when compared with that of Factor IXaα.

Introduction

Hemophilia B is a sex-linked hereditary bleeding disorder related to a plasma deficiency in functional blood coagulation Factor IX (1, 2). Plasma deficiencies in functional Factor IX result when there is a defect in the expression of the Factor IX gene or when the product of gene expression is structurally abnormal. Hemophilia B has been subclassified according to the plasma level of Factor IX antigen or cross-reacting material (CRM)[1] to antisera specific for Factor IX (3). In previous studies we investigated the structure/function of abnormal Factor IX isolated from the plasma of CRM^+ hemophilia B patients, i.e., patients with essentially normal plasma Factor IX antigen levels but significantly decreased Factor IX clotting activity (3). Factor $IX_{Chapel\ Hill}$, isolated from the plasma of a CRM^+ hemophilia B patient with a clinically mild bleeding tendency, has been studied in some detail (4–6). Factor $IX_{Chapel\ Hill}$ is activated by incubation with Factor XIa/calcium, but the rate of activation is somewhat slower than the rate of activation of normal Factor IX (5). In addition, the apparent clotting activity of activated Factor $IX_{Chapel\ Hill}$ is only 20% of the clotting activity of activated normal Factor IX.[2] Activation of Factor $IX_{Chapel\ Hill}$ was associated with the cleavage of only one (Arg180-Val181) of the two sites cleaved by Factor XIa/calcium during the activation of normal Factor IX (Arg145-Ala146, Arg180-Val181) (5). Recently we reported that the structural defect in Factor $IX_{Chapel\ Hill}$ is in the substitution of histidine for arginine at position 145 in the primary structure (6), which would appear to explain the inability of Factor XIa/calcium to cleave the protein at this site.

The cleavage/activation of bovine and human Factor IX by a protease present in Russell's viper venom (RVV-X) has been reported (7, 8). Cleavage of Factor IX by RVV-X/calcium occurs predominantly at one site (human Factor IX, Arg180-Val181; bovine Factor IX, Arg181-Val182) to produce Factor IXaα (M_r = 57,000). Factor IXaα is composed of two polypeptide chains, which are held together by disulfide bonds. The specific clotting activity of bovine Factor IXaα has been reported to be ~twofold lower than the clotting activity of bovine Factor IXaβ (7). Factor IXaβ is also composed of two polypeptide chains held together by disulfide bonds, but the additional cleavage of the protein at a second site (human Factor IX, Arg145-Ala146; bovine Factor IX, Arg146-Ala147) appears to release an activation peptide (M_r = 11,000), with a

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1. *Abbreviations used in this paper:* CRM, cross-reacting material; HPLC, high-performance liquid chromatography; k_{obs}, apparent first order rate constant for Factor X activation by activated Factor IX; PEG, polyethylene glycol; RVV, Russel's viper venom; RVV-X, a protease present in Russell's viper venom; TEA, triethanolamine; TosGlyProArgNaN, *N*α-*p*-tosyl-glycyl-L-prolyl-L-arginine-*p*-nitroanilide.

2. The term activated factor IX is used when the specific structure, i.e., bond cleavage sites, is not indicated. The terms Factor XIa/calcium and RVV/calcium indicate that Factor IX was activated by incubation with Factor XIa or RVV in the presence of calcium. The term Factor X/polylysine activity refers to Factor X activation by activated Factor IX in the presence of polylysine. The term clotting activity refers to the procoagulant activity of activated Factor IX in Factor IX deficient plasma.

concomitant decrease in the apparent molecular weight of the protein to ~45,000 (7–9). The specific clotting activities of human Factors IXaα and IXaβ have been reported to be essentially identical (8). This observation, however, is inconsistent with the finding that the specific clotting activity of activated Factor $IX_{Chapel\ Hill}$, which is structurally very similar to Factor IXaα, is only 20% of the clotting activity of Factor IXaβ (5).

Factor IXaα is cleaved to Factor IXaβ during prolonged incubation with RVV-X/calcium (8). This observation suggests that a given preparation of Factor IXaα would contain Factor IXaβ, which could increase the apparent clotting activity of the preparation. The present investigation was therefore undertaken to determine rigorously the relative clotting activities of activated Factor $IX_{Chapel\ Hill}$, Factor IXaα, and Factor IXaβ. Factor IXaα, prepared by incubation with Russell's viper venom (RVV)/calcium, was separated from Factor IXaβ by high-performance liquid chromatography (HPLC) before measurement of clotting activity. We have also measured the rates of Factor X activation by these enzymes in the presence of polylysine (10). This system was found to be very useful in measuring activated Factor IX concentrations by titration with antithrombin III and offered an approach for evaluating enzymatic activities in the absence of cofactors (calcium, phospholipid, Factor VIII) that are present in the clotting activity assay. We have found that the Factor X/polylysine activities of these enzymes are essentially identical, whereas the clotting activities of activated Factor $IX_{Chapel\ Hill}$ and Factor IXaα are only 20% of the clotting activity of Factor IXaβ.

Methods

Materials. Polylysine, M_r = 65,000 and RVV were purchased from Sigma Chemical Co. (St. Louis, MO). *N*α-*p*-Tosyl-glycyl-L-prolyl-L-arginine-*p*-nitroanilide (TosGlyProArgNaN) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bovine brain cephalin (Thrombofax) was purchased from Ortho Pharmaceutical (Raritan, NJ). Polyethylene glycol (PEG; M_r = 6,000) and triethanolamine (TEA) were purchased from Fisher Scientific Co. (Raleigh, NC).

Proteins. Factors IX and X were isolated from human plasma by a procedure incorporating various aspects of published procedures (11, 12). In brief, Factors IX and X were adsorbed from plasma with barium citrate. The proteins were eluted from the barium citrate with 40% ammonium sulfate. The ammonium sulfate eluate was dialyzed against 0.02 M sodium phosphate (pH 5.9), 0.1 M NaCl, 1.0 mM benzamidine and applied to a 2.5 × 30 cm DEAE-cellulose (DE-52; Whatman Chemical Separation, Inc., Clifton, NJ) column equilibrated with the dialysis buffer. Factors IX and X were eluted from the column with a 1 liter gradient from 0.1 to 0.55 M NaCl.

Factors IX and X (as well as prothrombin), which coelute from the DEAE-cellulose column, were separated from one another by dextran sulfate-agarose column chromatography as previously described (12). Effluent fractions from the dextran sulfate-agarose column containing Factors IX and X were separately pooled and concentrated by adsorption to and elution from a 1.0-cc bed volume DEAE-cellulose column. The preparations of Factor IX (200 U/mg) and Factor X (100 U/mg) were homogeneous as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (13). Prothrombin was not detected in the Factor IX or X preparations as measured by the generation of thrombin after prolonged incubation of samples with *Echnis carinatus* venom. Protein concentrations were determined spectrophotometrically at 280 nm by the use of extinction coefficient values of 1.33 and 1.16 ml mg^{-1} cm^{-1} for Factors IX and X, respectively (11). Factor $IX_{Chapel\ Hill}$ was isolated from hemophilia B patient plasma by the procedure described above for the isolation of normal Factor IX. Factor IX and X preparations were dialyzed overnight at 4°C against 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl. The dialyzed preparations were incubated for 30 min at room temperature with 10 mM diisopropylphosphofluoridate before storage at −70°C.

Human antithrombin III was isolated from barium citrate-adsorbed plasma by heparin-agarose affinity chromatography essentially as described previously (14). The preparation was judged to be homogeneous by SDS-polyacrylamide gel electrophoresis (13). Antithrombin III concentration was determined spectrophotometrically at 280 nm by means of an extinction coefficient value of 0.6 ml mg^{-1} cm^{-1} (14). When an M_r of 65,000 was used (14), 1 mol of purified antithrombin III was required to inactivate 1 mol of human α-thrombin in the presence or absence of heparin (15).

Human Factor XIa was partially purified from Celite (Celite 545; Johns-Manville Sales Corp., Denver, CO) activated human plasma as described previously (5). Factor XIa and RVV[3] were covalently coupled to agarose (Sepharose 4B) by the procedure described previously (16).

Measurement of activated Factor IX by plasma clotting activity. The clotting activities of solutions containing activated Factor IX were determined by the addition of 0.075-ml sample to a fibrometer cup containing 0.075 ml of nonactivated Factor IX deficient plasma and 0.075 ml Thrombofax. After the mixture was warmed to 37°C, 0.075 ml of 25 mM calcium chloride was added and the fibrometer was started. The clotting times of normal Factor IXaβ solutions, ranging in concentration from 2.5 to 250 nM (0.01125 to 11.25 μg/ml), were used to construct a standard curve. The clotting time of a given activated Factor IX solution was converted to the equivalent normal Factor IXaβ concentration from the standard curve. Therefore, activated Factor IX concentrations, determined by clotting activity, are expressed in micrograms or moles of normal Factor IXaβ per unit volume.

Measurement of activated Factor IX by Factor X/polylysine activity. Activated Factor IX was added to a solution containing 400 nM Factor X, 50 nM polylysine, 0.3 mM TosGlyProArgNaN, 0.1 M TEA (pH 8.0), 0.1 M NaCl, and 0.1% PEG. After the solution was mixed it was transferred to a 1.0-cm path length cuvette, which was then placed in a Beckman Acta CIII spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). The change in absorbance at 400 nm was measured as a function of incubation time at room temperature. The rate of change in absorbance per minute of incubation time (dA_{400}/dt) increased linearly with incubation time (t) as described by the following first order rate equation:

$$dA_{400}/dt = k_{obs} \cdot C \cdot t, \quad (1)$$

where k_{obs} is the apparent first order rate constant for Factor X activation by activated Factor IX, and C is the rate of change in A_{400} due to the hydrolysis of TosGlyProArgNaN by Factor Xa. Under the experimental conditions described above, the value of C was 1.374 × 10^7 (ODU/min)/M Factor Xa. This value was determined by the addition of a known amount of Factor Xa (RVV-agarose/calcium activated Factor X, active site titrated by the use of antithrombin III) to the buffer/polylysine/TosGlyProArgNaN solution and measurement of the initial rate of increase in A_{400}.

The absorbance (A_{400}) at any given incubation time was described by the integrated form of Eq. 1.

$$A_{400} = k_{obs} \cdot C \cdot t^2/2 + C' \cdot t + A^{t=0}_{400}. \quad (2)$$

In Eq. 2, C' is the rate of change in A_{400} due to the hydrolysis of TosGlyProArgNaN by Factor Xa (or other enzymes) present in the

3. Russell's viper venom, as obtained commercially, was used in the present study without fractionation to obtain the specific protease (RVV-X) responsible for Factor IX activation. The results of preliminary work with the unfractionated-venom and purified RVV-X were not measurably different in terms of the relative rates of cleavage of the Arg180-Val181 and Arg145-Ala146 bonds.

reaction solution at zero time. Since the value of C' was variable (see Results), for each series of assays a control was run in which buffer was added to the reaction solution instead of activated Factor IX. The absorbance of the control at incubation time t (A'_{400}), was therefore equal to $C' \cdot t + A^{t=0}_{400}$, where $A^{t=0}_{400}$ is the absorbance of the reaction solution at zero time. It is important to note that the addition of activated Factor IX to the reaction solution, in the absence of Factor X, did not have a measurable effect on the absorbance of the solution over the incubation times used in the present study. By substitution of A'_{400} into Eq. 2 and rearrangement of the equation, the value of k_{obs} could be determined at any incubation time according to the following equation:

$$k_{obs} = (A_{400} - A'_{400})2/C \cdot t^2. \quad (3)$$

For routine assay purposes, the final activated Factor IX concentration was varied up to a final concentration of ~50 nM in a total reaction volume of 0.5 ml. The incubation time was varied according to the concentration range of activated Factor IX in a given assay series. TosGlyProArgNaN hydrolysis was terminated by the addition of 0.5 ml of 50% acetic acid, and the absorbance was measured.

The absorbance values of the control and test solutions were substituted into Eq. 2 along with the incubation time for the assay series, and k_{obs} was calculated. The value of C was corrected for the dilution of the reaction solution due to the addition of acetic acid. Under these conditions, A_{400} values of <0.35 were proportional to the amount of Factor Xa formed during the incubation time. The k_{obs} values were proportional to the activated Factor IX concentration up to at least 50 nM and corresponded to a rate of Factor X activation of 0.035 mol Factor Xa/min per mol of activated Factor IX.

Measurement of activated Factor IX concentration by active site titration with antithrombin III. Activated Factor IX was added to a series of solutions containing from 50 to 250 nM antithrombin III, 0.01 mg/ml heparin, 0.1 M TEA (pH 8.0), 0.1 M NaCl, and 0.1% PEG. The final activated Factor IX concentration (assuming complete activation of a known concentration of Factor IX) was ~250 nM. The solutions were incubated at room temperature for 20 min after the addition of activated Factor IX. Samples (0.02 ml) were then removed and added to a solution (0.5 ml) containing 400 nM Factor X, 50 nM polylysine, 0.3 mM TosGlyProArgNaN, 0.1 M TEA (pH 8.0), 0.1 M NaCl, and 0.1% PEG. After incubation for 10 min at room temperature, 0.5 ml of 50% acetic acid was added. The absorbance at 400 nm of each solution was measured and the rate of Factor X activation (k_{obs}) was calculated according to Eq. 3. The rate of Factor X activation was plotted as a function of the antithrombin III concentration and the x-intercept value, corresponding to the concentration of antithrombin III required to neutralize completely the activated Factor IX, was determined by linear regression analysis of the data. Under the assumption that 1 mol of antithrombin III is required to neutralize 1 mol of activated Factor IX, the activated Factor IX concentration corresponded to the x-intercept value. The y-intercept values, corresponding to the rate of Factor X activation by the diluted activated Factor IX solution in the absence of antithrombin III, was then used to calculate the rate of Factor X activation per mol of activated Factor IX.

Preparation of activated Factor IX. Factor IX was added to a solution containing 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl, 7.0 mM $CaCl_2$, 0.1% PEG, and either Factor XIa-agarose or RVV-agarose. The final Factor IX concentration was ~400 nM (0.225 mg/ml). The amount of Factor XIa-agarose or RVV-agarose was adjusted such that Factor IX activation, as determined by Factor X/polylysine activity, was completed within 4 to 6 h of incubation at room temperature. Complete conversion of Factor IX to its activated forms was confirmed by SDS-polyacrylamide gel electrophoresis after sample reduction with β-mercaptoethanol. Incubation of normal Factor IX with Factor XIa-agarose/calcium resulted in conversion of the protein to the Factor IXaβ structure, whereas Factor $IX_{Chapel\ Hill}$ was converted to the Factor IXaα structure. Incubation of normal Factor IX with RVV-agarose/calcium resulted in conversion of the protein to the Factor IXaα structure predominantly, with a time dependent conversion (~30% in 6 h) to the Factor IXaβ structure (see Results). Prolonged incubation of normal Factor IX with RVV-agarose/calcium (~24 h) resulted in nearly quantitative conversion of the Factor IXaα to Factor IXaβ. Incubation of Factor $IX_{Chapel\ Hill}$ with RVV-agarose/calcium resulted in conversion of the protein to the Factor IXaα structure only. The concentration of activated Factor IX in a given solution was determined by active site titration with antithrombin III.

Size exclusion chromatography of activated Factor IX. Factor IXaβ could be separated from Factor IXaα and unactivated Factor IX by HPLC with Spherogel-TSK 3000 SW column (7.5 mm × 30 cm; Altex Scientific, Inc., Berkeley, CA). The running buffer was 0.05 M Tris/HCl (pH 7.0), 0.5 M NaCl, and the flow rate was 0.5 ml/min. In a typical experiment, 0.2-ml sample containing 0.225 mg/ml of protein was injected and a 5.5-ml fraction was collected before the collection of 0.12-ml fractions. Essentially 100% of the protein, as determined by activity assays, was recovered in all experiments. The separation of Factor IXaβ from Factor IXaα (and Factor IX) was somewhat better than expected strictly on the basis of the molecular weight differences. The elution volume of Factor IXaα (7.3 ml) corresponded to an apparent molecular weight of 80,000 (actual molecular weight, 57,000), whereas the elution volume of Factor IXaβ (8.3 ml) corresponded to an apparent molecular weight of 45,000, which is the actual molecular weight of Factor IXaβ.

Results

Factor X activation by activated Factor IX in the presence of polylysine. Factor IXaβ, prepared by incubation of normal Factor IX with Factor XIa-agarose/calcium, was added to a solution containing Factor X, polylysine, and TosGlyProArgNaN, and the absorbance of the solution at 400 nm was followed spectrophotometrically over a 10-min incubation time. A similar experiment was run in which buffer was added instead of Factor IXaβ. In the presence of Factor IXaβ, the absorbance increased nonlinearly with incubation time, whereas in the absence of Factor IXaβ, the absorbance increased linearly. These results are shown in Fig. 1. The rate of change in absorbance (ΔA_{400}) per minute of incubation time in the presence of Factor IXaβ was plotted as a function of incubation time. As shown in the inset in Fig. 1, the ΔA_{400} per minute values increased linearly with incubation time which, according to Eq. 1, indicated that the activation of Factor X by Factor IXaβ in the presence of polylysine followed first order kinetics over the 10-min incubation. From the slope of the line (Fig. 1, inset), k_{obs} was calculated according to Eq. 1 to be equal to 0.30 nM Factor Xa/min. The absorbance values at t = 10 min for both experiments (A_{400} = 0.365, A'_{400} = 0.135) were used in Eq. 3 to calculate the k_{obs}; 0.30 nM Factor Xa/min. It is apparent that either method of calculation gives equivalent values for k_{obs}.

The linear increase in absorbance observed in the absence of Factor IXaβ suggested that trace amounts of Factor Xa were present in the Factor X preparation. For the experiment shown in Fig. 1, the rate of increase in absorbance was 0.0135 ODU/min, which corresponds to a Factor Xa concentration of ~1.0 nM. Since the Factor X concentration in the experiment was 400 nM, only 0.25% of the preparation appeared to be activated. The amount of Factor Xa present in the Factor X prepartion was variable, however, and appeared to increase with storage at 4°C. Since the Factor X preparation, treated with diisopropylfluorophosphate immediately after isolation, was initially devoid of amidolytic activity, it appeared that a

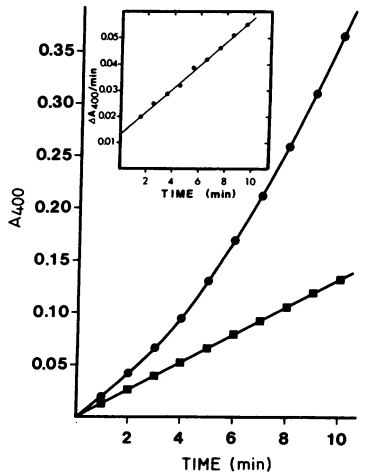

Figure 1. Synthetic substrate hydrolysis vs. Factor IXaβ/Factor X incubation time in the presence of polylysine. Factor IXaβ was added to a solution containing 400 nM Factor X, 50 nM polylysine, 0.3 mM TosGlyProArgNaN, 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG, and the absorbance at 400 nm (A_{400}) was monitored spectrophotometrically (•). The final factor IXaβ concentration was 8.65 nM. The experiment was repeated in the absence of Factor IXaβ (■). (*Inset*) The ΔA_{400}/min values obtained in the presence of activated Factor IX were calculated over 1-min time increments and are plotted against the incubation time.

protease able to activate Factor X in the absence of calcium was a contaminant of the preparation. The protease is not susceptible to inhibition by diisopropylfluorophosphate or able to hydrolyze TosGlyProArgNaN. Storage of the Factor X preparation at −70°C greatly decreased Factor X activation; therefore, freshly thawed Factor X solutions were used in all subsequent experiments. A control, however, was run for each assay series.

The rates of activation of Factor X in the presence of polylysine by Factor IXaβ and activated factor $IX_{Chapel\ Hill}$ were not measurably different. Solutions containing approximately equal concentrations of both enzymes were titrated with antithrombin III to determine the active site concentrations. As shown in Fig. 2, the residual Factor X/polylysine activities of the solutions decreased linearly with increasing antithrombin III concentration. On the basis of the amount of antithrombin III that was required to inhibit completely the Factor X/polylysine activity of the enzyme solution, the initial concentrations of Factor IXaβ and activated Factor $IX_{Chapel\ Hill}$ were 225 and 295 nM, respectively. For both enzymes, the rate of Factor X activation was 0.035 mol Factor Xa/min per mol of active sites.

Factor IX activation by RVV-agarose/calcium. Incubation of normal Factor IX with RVV-agarose/calcium was associated with an increase in Factor X/polylysine and clotting activities. A significant difference between the apparent activated Factor IX concentrations as determined by the two activity assays was observed, however. These results are shown in Fig. 3. The activated Factor IX concentration, measured by Factor X/polylysine activity (0.225 mg/ml), appeared to indicate complete activation of Factor IX after >4 h of incubation. The activated Factor IX concentration, measured by clotting activity, increased continually over the 6-h incubation. Since the activated Factor IX concentration, determined by both activity assays, is based on the activity of Factor IXaβ (i.e., Factor IXaβ was used to construct the standard curves used to determine the activated Factor IX concentration), the results suggest that the predominant form of activated Factor IX obtained by incubation of Factor IX with RVV-agarose/calcium, i.e., Factor IXaα, has significantly lower clotting activity than does Factor IXaβ. The factor X/polylysine activities of Factors IXaα and IX appear to be essentially equivalent. Under the assumption that the Factor X/polylysine activity measured concentration, the clotting activity of Factor IXaα is <20% of the clotting activity of Factor IXaβ.

Samples taken at timed intervals from the Factor IX/RVV-agarose/calcium incubation mixture were fractionated by HPLC. As shown in Fig. 4, after incubation for 2 h, essentially

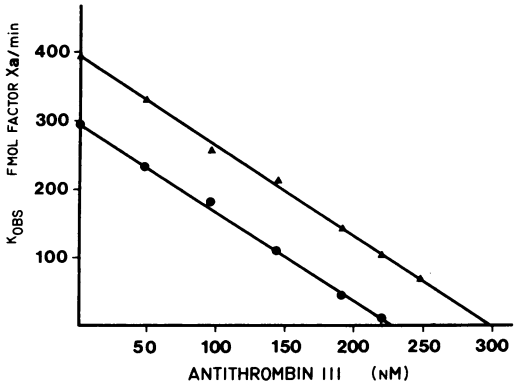

Figure 2. Determination of the activated Factor IX concentration by titration with antithrombin III. Antithrombin III was added to a solution containing approximately equal amounts of either Factor IXaβ (•) or activated Factor $IX_{Chapel\ Hill}$ (▲), 0.01 mg/ml heparin, 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG, and incubated for 20 min at room temperature. Samples (0.02 ml) were removed and added to a solution (0.5 ml) containing 400 nM Factor X, 50 nM polylysine, 0.3 mM TosGlyProArgNaN, 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG, and the rate of Factor X activation was determined as described in Methods.

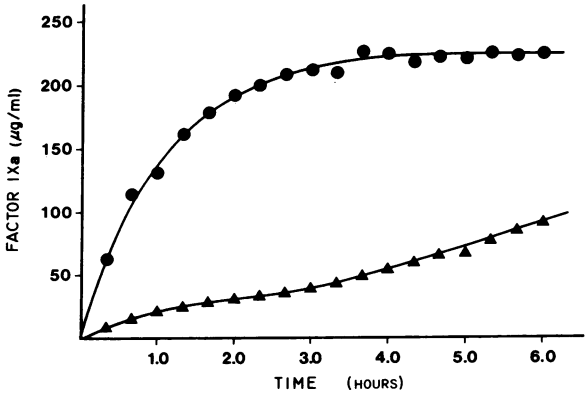

Figure 3. Factor IX activation by RVV-agarose/calcium. RVV-agarose was added to a solution containing normal Factor IX (0.225 mg/ml), 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl, 0.1% PEG, 7.0 mM $CaCl_2$, and incubated at room temperature. At timed intervals samples were removed and assayed for activated Factor IX by Factor X/polylysine activity (•) and clotting activity (▲) as described in Methods. The amount of activated Factor IX was determined from standard curves, which related either Factor X/polylysine activity or clotting activity to normal Factor IXaβ concentration.

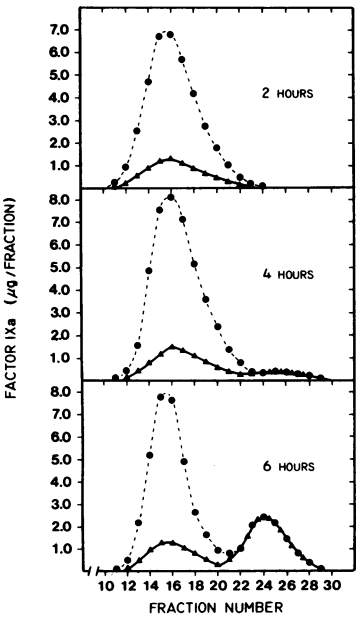

Figure 4. High-performance size exclusion chromatography of RVV-agarose-activated normal Factor IX. Factor IX was incubated with RVV-agarose/calcium as described in the legend to Fig. 3. At timed intervals, 0.2-ml samples were removed and chromatographed by HPLC as described in Methods. After a 5.5-ml fraction was collected (not shown), 0.12-ml fractions were collected and assayed for activated Factor IX by Factor X/polylysine activity (●) and by clotting activity (▲) as described in Methods.

all of the Factor X/polylysine activity was present in a single peak. By 4 h, however, a second peak of Factor X/polylysine activity could be distinguished, which, by 6 h of incubation, was quite pronounced. The ratio of clotting activity to Factor X/polylysine activity in the first peak was 0.17, whereas the ratio in the second peak was essentially 1.0. These results indicate that the increase in clotting activity observed with prolonged incubation of Factor IX with RVV-agarose/calcium (Fig. 3) is associated with the conversion of Factor IXaα to a lower apparent molecular weight form of Factor IX, which is as active as Factor IXaβ in the clotting and Factor X/polylysine activity assays.

The second peak of Factor X/polylysine activity obtained with RVV-agarose/calcium activated Factor IX (Fig. 4) eluted from the HPLC column in the same volume as the Factor X/polylysine activity in Factor XIa-agarose/calcium activated Factor IX, i.e., Factor IXaβ. These results are shown in Fig. 5. Samples of RVV-agarose/calcium activated Factor IX were subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Most of the RVV-agarose/calcium activated Factor IX migrated at the same rate as unactivated Factor IX, and a small amount of the protein migrated at the same rate as Factor IXaβ (not shown). A sample of activated Factor IX obtained after prolonged incubation with RVV-agarose/calcium was analyzed for amino terminal amino acid sequences. On three successive sequencer cycles, the following amino acids were identified: (*a*) Tyr, Val, Ala; (*b*) Asn, Val, Glu; and (*c*) Ser, Gly, Thr. These results are consistent with the cleavage of Factor IX at the Arg145-Ala146 and Arg180-Val181 bonds, which would generate the light chain (Tyr-Asn-Ser), heavy chain (Val-Val-Gly), and activation peptide (Ala-Glu-Thr) sequences observed with Factor IXaβ (8).

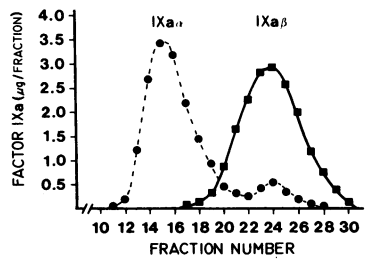

Figure 5. High-performance size exclusion chromatography of activated Factor IX. Normal Factor IX (0.145 mg/ml) was incubated with either RVV-agarose (●) or Factor XIa-agarose (■) in a solution containing 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl, 0.1% PEG, 7.0 mM $CaCl_2$. After ~2 h, the amount of activated Factor IX, as determined by Factor X/polylysine activity, in both incubation mixtures was 0.145±0.01 mg/ml. Samples (0.1 ml) were then chromatographed and assayed for activated Factor IX activity as described in the legend to Fig. 4.

The concentrations of various activated Factor IX solutions were determined by both Factor X/polylysine activity and clotting activity. The ratio between the concentrations determined by clotting activity and Factor X/polylysine activity was defined as the relative activity of the activated Factor IX preparation. The relative activity of Factor IXaβ was 1.0 according to this definition. The relative activities of Factor IXaα (0.17) and activated Factor $IX_{Chapel\ Hill}$ (0.19) were essentially identical. These results are summarized in Table I.

Discussion

Activated Factor $IX_{Chapel\ Hill}$ and Factor IXaα are structurally very similar. Both enzymes are formed by cleavage of a single peptide bond (Arg180-Val181), which produces molecules consisting of two polypeptide chains held together by disulfide bond(s) (5, 8). The carboxyl-terminal polypeptide chain (235 residues) of activated Factor $IX_{Chapel\ Hill}$ and Factor IXaα corresponds to the heavy chain of Factor IXaβ and contains the active site residues of the enzymes. The amino-terminal polypeptide chains (180 residues) consist of the γ-carboxyglutamic acid residue-containing light chain of Factor IXaβ plus the activation peptide which is produced when Factor IXaβ is formed. The primary structural difference between activated Factor $IX_{Chapel\ Hill}$ and Factor IXaα appears to be the substitution of histidine for arginine at position 145 (6).

In an earlier report we suggested that the enzymatic properties of activated Factor $IX_{Chapel\ Hill}$ and Factor IXaα should be similar (6). In the present study we have shown that the clotting activities of activated Factor $IX_{Chapel\ Hill}$ and Factor IXaα are very similar but <20% of the clotting activity of Factor IXaβ. We conclude from this observation that the clotting activity of activated Factor $IX_{Chapel\ Hill}$ is abnormal, as compared with that of Factor IXaβ. This further suggests that the substitution of histidine for arginine at position 145 in the primary structure of Factor $IX_{Chapel\ Hill}$, while severely limiting cleavage at this site by Factor XIa/calcium, does not have a

Table I. Concentrations of Activated Factor IX Determined by Factor X/Polylysine and Clotting Activities

Factor IXa	Factor X/polylysine activity	Clotting activity	Relative activity
[Factor IXaβ]	2.25 μM	2.25 μM	1.0
[Factor IXaα]	2.79 μM	0.48 μM	0.17
[Factor IXa_{CH}]	2.95 μM	0.56 μM	0.19

Activated Factor IX solutions were assayed for Factor X/polylysine and clotting activities and the activities were converted to the corresponding concentrations from standard curves as described in Methods. The concentration of activated Factor IX determined by clotting activity was divided by the concentration determined by Factor X/polylysine activity to obtain the relative activity value. Factor IX_{CH}, Factor $IX_{Chapel\ Hill}$.

measurable effect on the clotting activity relative to Factor IXaα.

Activation of human Factor IX by incubation with RVV-agarose/calcium has been shown to be associated with the rapid cleavage of the Arg180-Val181 peptide bond, but additional cleavage has been observed (8). In the present study we have shown that two enzyme forms of Factor IXa are produced by RVV-agarose/calcium cleavage of Factor IX. The predominant enzyme produced was Factor IXaα whereas the second enzyme was indistinguishable from Factor IXaβ, as suggested previously (8). In our initial attempts to prepare Factor IXaα we used much larger amounts of RVV-agarose than in the present report. Although this greatly decreased the incubation time required to activate the Factor IX, we found that the clotting activity rapidly approached the clotting activity expected for Factor IXaβ, as reported by DiScipio and co-workers (8). In view of the results obtained in the present study it appears that the formation of Factor IXaβ could account for the apparently similar clotting activities of Factors IXaα and IXaβ when Factor IX is activated by larger amounts of RVV-agarose.

The clinical bleeding tendency of the patient with hemophilia B Chapel Hill is very mild. Only under conditions where there is a major challenge to the hemostatic process, e.g. surgery, does the patient appear to have significant bleeding. Since Factor $IX_{Chapel\ Hill}$ is activated to an essentially normal Factor IXaα molecule, several interesting possibilities about the role of Factor IX in normal hemostasis can be considered. For example, it has been shown that <2% of the Factor IX present in normal plasma is activated when plasma clots in vitro (17). If Factor IX activation is not the rate limiting step during hemostasis, then enough Factor $IX_{Chapel\ Hill}$ may be activated for relatively normal hemostasis to occur. Alternatively, the recent work of Link and Castellino has shown that the differences between the enzymatic properties of bovine Factors IXaα and IXaβ can be related to differences in the interactions of these enzymes with cofactors (18). Normal hemostasis probably presents activated Factor IX with cofactor concentrations which are considerably different from those encountered in an in vitro clotting activity assay. Therefore, the enzymatic properties of activated Factor $IX_{Chapel\ Hill}$ may be essentially equal to those of Factor IXaβ under the conditions encountered during normal challenge to the hemostatic process. Under the conditions encountered during major challenge to the hemostatic process, however, the defect in Factor $IX_{Chapel\ Hill}$ results in a significant bleeding diathesis.

The present study has shown that activated Factor $IX_{Chapel\ Hill}$, Factor IXaα, and Factor IXaβ activate Factor X at similar rates in the presence of polylysine. This observation is interesting in that it indicates that there are conditions under which the three enzymes are essentially equally active. The mechanism of action of polylysine in accelerating the rate of Factor X activation by activated Factor IX is not known, however, and it is difficult to compare rates of Factor X activation in the presence of polylysine with rates of activation observed in the presence of cofactors (calcium, phospholipid, Factor VIIIa). In an earlier study we observed a rate of Factor X activation of 20.5 mol Factor Xa/min per mol Factor IXaβ in the presence of 5 U/ml Factor VIIIa and optimal levels of calcium and phospholipid (19). This rate is obviously much greater (~600-fold) than the rate of Factor X activation in the presence of polylysine observed in the present study. Although this suggests that polylysine is not as effective as calcium, phospholipid, Factor VIIIa in promoting Factor X activation, the conditions for Factor X activation in the presence of polylysine used in the present study may not have been optimal. In any event, the similar rates of Factor X activation in the presence of polylysine by the three enzymes studied herein suggests that the enzymes are structurally/functionally similar with respect to interactions with Factor X and polylysine, whereas cofactor interactions, as measured by relative clotting activities, appear to differ significantly. Furthermore, these results appear to indicate that the active site regions of the three enzymes are similar with respect to the Factor X binding domain and the active sites residues required for the cleavage of Factor X. Determination of the mechanism of action of polylysine may reveal structural properties of activated Factor IX that are important for cofactor (calcium, phospholipid, Factor VIIIa) interactions and a better understanding of the structure/function of Factor IX.

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