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Research Article

The kinetics of activation of human Factor IX by human Factor XIa was studied by measuring the release of a trichloroacetic acid-soluble tritium-labeled activation peptide from Factor IX by a modification of a method described for bovine Factor IX activation by Zur and Nemerson (Zur, M., and Y. Nemerson, 1980, J. Biol. Chem., 255:5703-5707). Initial rates of trichloroacetic acid-soluble 3H-release were linear over 10-30 min of incubation of Factor IX (88 nM) with CaCl2 (5 mM) and with pure (greater than 98%) Factor XIa (0.06-1.3 nM), which was prepared by incubating human Factor XI with bovine Factor XIIa. Release of 3H preceded the appearance of Factor IXa activity, and the percentage of 3H released remained constant when the mole fraction of 3H-labeled and unlabeled Factor IX was varied and the total Factor IX concentration remained constant. A linear correlation (r greater than 0.98, P less than 0.001) was observed between initial rates of 3H-release and the concentration of Factor XIa, measured by chromogenic assay and by radioimmunoassay and added at a Factor IX:Factor XIa molar ratio of 70-5,600. Kinetic parameters, determined by Lineweaver-Burk analysis, include Km (0.49 microM) of about five- to sixfold higher than the plasma Factor IX concentration, which could therefore regulate the reaction. The catalytic constant (kcat) (7.7/s) is approximately 20-50 times higher than that reported by […]

Kinetics of the Factor XIa Catalyzed Activation of Human Blood Coagulation Factor IX

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Abstract. The kinetics of activation of human Factor IX by human Factor XIa was studied by measuring the release of a trichloroacetic acid-soluble tritium-labeled activation peptide from Factor IX by a modification of a method described for bovine Factor IX activation by Zur and Nemerson (Zur, M., and Y. Nemerson, 1980, *J. Biol. Chem.*, 255:5703–5707). Initial rates of trichloroacetic acid-soluble ^{3}H-release were linear over 10–30 min of incubation of Factor IX (88 nM) with $CaCl_2$ (5 mM) and with pure (>98%) Factor XIa (0.06–1.3 nM), which was prepared by incubating human Factor XI with bovine Factor XIIa. Release of ^{3}H preceded the appearance of Factor IXa activity, and the percentage of ^{3}H released remained constant when the mole fraction of ^{3}H-labeled and unlabeled Factor IX was varied and the total Factor IX concentration remained constant. A linear correlation ($r > 0.98$, $P < 0.001$) was observed between initial rates of ^{3}H-release and the concentration of Factor XIa, measured by chromogenic assay and by radioimmunoassay and added at a Factor IX:Factor XIa molar ratio of 70–5,600. Kinetic parameters, determined by Lineweaver-Burk analysis, include a K_m (0.49 μM) of about five- to sixfold higher than the plasma Factor IX concentration, which could therefore regulate the reaction. The catalytic constant (k_{cat}) (7.7/s) is ~20–50 times higher than that reported by Zur and Nemerson (Zur, M., and Y. Nemerson, 1980, *J. Biol. Chem.*, 255:5703–5707) for Factor IX activation by Factor VIIa plus tissue factor. Therefore, depending on the relative amounts of Factor XIa and Factor VIIa generated in vivo and other factors which may influence reaction rates, these kinetic parameters provide part of the information required for assessing the relative contributions of the intrinsic and extrinsic pathways to Factor IX activation, and suggest that the Factor XIa catalyzed reaction is physiologically significant.

Introduction

Human Factor IX is essential for normal blood coagulation in vitro and for normal hemostasis in vivo (1). It is present as a zymogen with a molecular weight of 57,500 at a concentration of 2.6–5 μg/ml in human plasma (2–4). Problems that arise in the quantitative analysis of Factor IX activation include the lack of specificity of coagulation assays and the lack of availability of chromogenic substrates specifically cleaved by Factor IXa. Therefore, Berkowitz and Nemerson (5) have developed a radiometric assay for activated bovine Factor IX similar to that described for bovine Factor X by Silverberg et al. (6). This assay takes advantage of the fact that bovine Factor IX is a single chain glycoprotein containing 25% carbohydrate which is cleaved during activation at an arginine-alanine bond and at an arginine-valine bond to release a 10,000 D activation peptide that contains ~50% of the carbohydrate residues in the molecule (7, 8). Since these carbohydrate residues can be radiolabeled with tritium in the parent molecule and the activation peptide remains soluble in 5% trichloroacetic acid (TCA) whereas the remainder of the molecule is insoluble in TCA, Factor IX activation can be assayed as release of the activation peptide.

The conversion of Factor IX to an active enzyme can occur by proteolytic cleavage of the zymogen either by Factor XIa as a result of activation of the contact system of intrinsic coagulation (2, 4, 9) or by the Factor VII-tissue factor pathway of extrinsic coagulation (10–12). Since deficiencies of both Factor XI and Factor VII can cause hemostatic defects, it is important to define kinetically the relative contributions of these two pathways for initiating intrinsic coagulation. The objectives of the present study therefore were, first, to validate the Factor IX activation peptide release assay as a measure of human Factor IX activation

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and of Factor XIa enzymatic activity, and secondly, to determine kinetic parameters for the activation of human Factor IX by human Factor XIa.

Methods

Materials. All common laboratory reagents and buffers were reagent grade or the highest quality available and were purchased from Sigma Chemical Co., St. Louis, MO; Fisher Chemical Co., Fairlawn, NJ; or from J. T. Baker Chemical Co., Phillipsburg, NJ. Betaphase scintillation fluid was obtained from West Chem Products, San Diego, CA. Benzamidine hydrochloride, soybean trypsin inhibitor, sodium heparin, and cephalin (rabbit brain extract) were purchased from Sigma Chemical Co. Diethylaminoethyl (DEAE) cellulose (DE52) was obtained from Whatman Chemical Separation, Inc., Clifton, NJ. Sulfated dextran was synthesized with reagents and methods as described by Miletich et al. (13). Plasmas deficient in Factors IX, X, and XI or prothrombin were obtained from George King Biomedical, Overland Park, KA. Acrylamide, sodium dodecyl sulfate (SDS), *N,N'*-methylene-*bis*-acrylamide, *N,N,N',N'*-tetramethylethylenediamine, and cyanogen bromide-activated Sepharose were purchased from Bio-Rad Laboratories, Richmond, CA. Tritiated sodium borohydride of two different specific activities (5–15 Ci/mmol and 72–83 Ci/mmol) was purchased in crystalline form in sealed ampoules from New England Nuclear, Boston, MA. The chromogenic substrate Pyr-Gly-Pro-Arg-paranitroanilide 2 HCl (S-2366) was a gift to Dr. R. W. Colman from AB KABI Peptide Research, Molndal, Sweden.

Purification of proteins. Factor XI was purified from human plasma by a modification of the method of Bouma and Griffin (14). Essentially 1 liter of normal human plasma obtained by plasmapheresis was chromatographed on a DEAE-Sephadex A-50 column at pH 8.3. The void volume fraction containing Factor XI was subjected to SP-Sephadex G-50 chromatography at pH 5.3, followed by affinity column chromatography using insolubilized high molecular weight (M_r) kininogen (15). High M_r kininogen was purified by the method of Kerbiriou and Griffin (16) and was insolubilized on cyanogen bromide-activated Sepharose supports utilizing instructions provided by Bio-Rad Laboratories. Pooled fractions eluted from the SP-Sephadex G-50 column were dialyzed into 0.04 M Tris, 15 M NaCl, pH 7.4, applied to a 1.4 × 2 cm high M_r kininogen column, and eluted with 0.2 M sodium acetate, 0.6 M NaCl, pH 5.5. The Factor XI thereby purified appeared homogenous on polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1) and had a specific activity of 270 U/mg 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 apparent homogeneity by alkaline gel electrophoresis utilizing modifications of methods described by DiScipio et al. (2) and by Miletich et al. (13), and employing as starting material human Factor IX concentrate (Konyne; Cutter Laboratories, Berkeley, CA), kindly provided by Dr. Milton M. Mozen and Dr. Michael H. Coan. The vitamin K-dependent coagulation proteins were adsorbed to barium citrate, eluted, dialyzed, and applied to a DEAE-cellulose column from which Factor IX, Factor X, and prothrombin were eluted as described (2). The fractions containing these proteins were pooled, dialyzed, and applied to a sulfated dextran column which separates prothrombin, Factor IX, and Factor X (13). Final purification of Factor IX to apparent homogeneity (Fig. 1) was accomplished by preparative polyacrylamide alkaline slab gel electrophoresis (17). The gel zone containing Factor IX was identified by staining 0.5 cm wide guide strips from the center and either ends of the preparative slab gel, which was cut out and eluted electrophoretically as previously described (18). The purified protein (185–225 U/mg, sp. act.) was stored in 0.012 M Tris, 0.045 M glycine, pH 8.3 at −185°C.

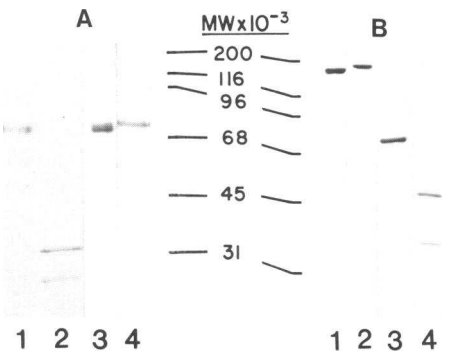

Figure 1. Polyacrylamide slab gel electrophoresis of purified proteins. (*A*) 10% SDS gels of cold and radiolabeled purified Factor IX (3–4 μg applied to each lane): lane 1, reduced radiolabeled Factor IX; lane 2, reduced radiolabeled Factor IXa; lane 3, unreduced cold Factor IX; lane 4, reduced cold Factor IX. (*B*) 7.5% SDS gels of purified Factor XI (lanes 1 and 3, 4–5 μg applied to each) and Factor XIa (lanes 2 and 4, 4–5 μg applied to each): lanes 1 and 2, unreduced; lanes 3 and 4, reduced. MW, molecular weight.

Activation of Factor XI. Purified Factor XI (270 U/mg, sp. act.) was activated by incubation with bovine Factor XIIa, kindly provided by Dr. Edward P. Kirby. To 100 μl (100 μg) of purified Factor XI (in 0.2 M acetate buffer, pH 5.3), concentrated Tris base was added to bring pH to 7.5 and this was followed by 6 μl (3 μg) of two-chain bovine Factor XIIa, which hydrolyzed 3.78×10^{-9} mol of the chromogenic substrate S2302/min per μg protein at pH 8.0 and 37°C. The proteins were incubated at 37°C and the incubation mixture was tested at intervals for Factor XIa coagulant activity and for proteolytic cleavage by reduced SDS-gel electrophoresis. The appearance of maximal coagulant activity occurred at 2 h of incubation at which time >95% of the protein was present in cleavage products of M_r of 50,000 and 30,000 (Fig. 1).

Tritium labeling of Factor IX. The entire procedure, including oxidation, reduction, and all dialyses, was carried out in a septum-capped dialysis bag by a modification of the method described by Van Lenten and Ashwell (19) and adapted for bovine Factor X by Silverberg et al. (6). In a typical labeling, ~500 μl (2.5 mg/ml or 45 μM) of the Factor IX fraction from the sulfated dextran column was dialyzed for 4 h at 4°C against 0.1 M NaCl, 0.1 M acetate buffer, pH 5.8, and then was oxidized for 10 min at 4°C with sodium periodate at concentrations given under Results. The oxidation reaction was quenched by the addition of ethylene glycol at a molar concentration 20-fold greater than that of the sodium periodate used in the oxidation step. The sample was then dialyzed continuously overnight at 4°C against 0.1 M NaH_2PO_4, pH 7.0. Reduction of the oxidized carbohydrate groups was then accomplished by the addition of tritiated 2.7 mM sodium borohydride dissolved in 50 mM NaOH at 4°C, followed by 4.5 mM unlabeled $NaBH_4$ to assure complete reduction. Initially, the tritiated borohydride preparation used had a specific radioactivity of 5–15 Ci/mmol. In subsequent experiments, tritiated borohydride with a higher specific activity (72–83 Ci/mmol) was used. Removal of unbound $NaBH_4$ was accomplished by continuous dialysis for 3 d against 150 mM NaCl, 50 mM Tris, 2.5 mM EDTA, pH 7.4. The labeled protein was finally purified by alkaline gel electrophoresis as indicated above. The gel-purified, labeled protein was homogeneous as judged by fluorography (20) of 7.5% SDS-poly-

acrylamide slab gels, and >98% of the radioactivity was precipitable in 5% TCA. Radiolabeled Factor IX (230 μg/ml, Fig. 1, part *A*, lane 1) was activated with 2.3 μg/ml Factor XIa in Hepes buffer containing 5 mM $CaCl_2$ for 2 h at 37°C, and the SDS-gel profiles of the resulting Factor IXa are shown in part *A*, lane 2 of Fig. 1. The heavy and light chains of Factor IXa are visualized but not the activation peptide; this is probably because the activation peptide washes out the gel during the fixing and staining procedure.

Radiometric assay of Factor IX activation. Tritiated Factor IX (475,000 cpm/μg) which retained >90% of its coagulant activity was incubated at 37°C with 5 mM $CaCl_2$ (determined to be the optimal concentration) and with Factor XIa, and the release of ^3H soluble in 5% TCA was measured essentially as described previously for bovine Factor X (6) and for bovine Factor IX (5). Assays were carried out in Tris (50 mM), NaCl (100 mM), pH 7.5, (Tris-buffered saline [TBS][1]) containing bovine serum albumin (1 mg/ml) in reaction volumes of 300–500 μl. At various time intervals, 80 μl samples were removed and added to 240 μl of an ice-cold mixture containing one part TBS and two parts 50 mM EDTA, pH 7.5. To this mixture was added 160 μl of ice-cold 15% TCA or TBS. The 400-μl capped polypropylene microfuge tubes (Eppendorf) containing this mixture were kept on ice and vortexed vigorously and repeatedly for 2 min, and then immediately centrifuged at 10,000 *g* for 3 min in a bench top Brinkman Model 3200 microfuge (Brinkmann Instruments, Inc., Westbury, NY). Thereafter, 100-μl aliquots of the supernatants were removed in triplicate into 10 ml of scintillation fluid and counted for ^3H in a Beckman LS 8000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Functional assays of coagulation proteins. Factors IX, X, and XI were assayed utilizing minor modifications of the kaolin-activated partial thromboplastin time (21) with appropriate congenitally deficient substrate plasmas. Factor XIa activity and Factor IXa activity were estimated utilizing a similar assay carried out in polystyrene tubes without the addition of kaolin. To measure the relative amount of Factor IXa generated in time course experiments, purified Factor IX (0.17 μM or 10 μg/ml) was activated by incubation (37°C) with Factor XIa (25 nM or 0.4 μg/ml) and $CaCl_2$ (5 mM) to full extent, as assessed by complete proteolytic cleavage by SDS-gel electrophoresis and by development of full coagulant activity in the Factor IXa clotting assay. A standard curve was made by serial dilution of the Factor IXa in TBS and plotting the logarithm of clotting time vs. the logarithm of the protein concentration. This standard curve was used to assay the Factor IXa generated at various times of incubation of Factor IX with Factor XIa.

Factor XIa was also assayed for amidolytic activity by using the chromogenic substrate, Pyr-Glu-Pro-Arg-*p*-nitroanilide (S-2366), as described by Scott et al. (22). Factor XIa (10 μl), at concentrations of 0.05–5.0 μg/ml (0.3–30 nM), as determined by radioimmunoassay was added to a prewarmed (37°C) cuvette containing 270 μl 0.1 M Na phosphate, pH 7.6, 0.15 M NaCl, 1 mM EDTA, and 30 μl of S-2366 (11.5 mM; final concentration, 1.11 mM). The change in absorbance was recorded at 405 nm and the Factor XIa amidolytic activity was calculated by comparison with a standard curve prepared by activating various dilutions of pooled normal plasma as described (22). By comparing the results of the amidolytic assay of purified Factor XIa (127 U/ml) with those of the Factor XI coagulation assays for purified Factor XIa (135 U/ml) and with the radioimmunoassay for Factor XI (750 μg/ml), we were able to determine a specific activity for our Factor XIa preparation of 180 U/mg protein. The fact that essentially all the Factor XI was in the proteolytically cleaved form (Factor XIa) was confirmed by reduced SDS-polyacrylamide gel profiles (Fig. 1).

Radioimmunoassay of Factor XI or XIa. Antibody to purified human Factor XI, raised in rabbits by three weekly injections of 80–100 μg of protein in a 1:1 emulsified mixture with Freund's adjuvant and by a subsequent booster injection 7–10 d before bleeding, gave a single precipitin band with purified Factor XI, and at a 1:100 dilution, abolished the Factor XI coagulant activity of a 1:10 dilution of normal pooled human plasma. Preimmune rabbit serum was negative on all double immunodiffusion tests and did not inhibit the Factor XI clotting activity of normal human plasma. Purified Factor XI was radiolabeled to a specific radioactivity of 500,000 cpm/μg with ^{125}I by the procedure of Bolton and Hunter (23), and the ^{125}I-labeled Factor XI was separated from unbound ^{125}I as previously described (24). The radioimmunoassay of Factor XI antigen was carried out by incubating 25 μl of purified Factor XI or unknown sample and 25 μl of ^{125}I-labeled Factor XI for 90 min at 37°C in Eppendorf microcentrifuge tubes with narrow bore extended tips. Thereafter, 30 μl of 10% Staphylococcus protein A bacterial adsorbant, prepared as described by Kessler (25), was added, mixed, and incubated for an additional 30 min at room temperature. The tubes were then centrifuged for 2 min in a Beckman Model B microfuge (Beckman Instruments, Inc.), the tips amputated, and the supernatant and pellets counted separately in an Intertechnique Model CG4000 gamma counter (Intertechnique, Plaisir, France). A linear relationship was demonstrated between the percentage of bound radioactivity and the logarithm of the concentration of unlabeled Factor XI or Factor XIa added at concentrations between 0.01–0.2 μg/ml (0.06–1.2 nM). The assay did not distinguish between Factor XI and Factor XIa and could be used to measure either protein.

Protein analysis. Protein assays were performed according to the method of Lowry et al. (26). Polyacrylamide slab gel electrophoresis in SDS was carried out according to the procedure of Laemmli (27). Autofluorography of gels of tritium-labeled Factor IX was carried out as described by Bonner and Laskey (20).

Calculations of kinetic constants. The derivation of kinetic constants for the Factor XIa catalyzed activation of Factor IX was based on a one enzyme, one substrate model. Values for Michaelis constant (K_m) and maximum velocity (V_{max}) were obtained by the Lineweaver-Burk method (28), with regression lines on double reciprocal plots drawn by eye. The catalytic constant (k_{cat}) was calculated by dividing V_{max} by the enzyme concentration. The coefficient of proteolytic efficiency was defined as k_{cat}/K_m (29). Alternatively, when kinetic constants were calculated using least-squares fit and the FORTRAN program of Cleland (30) with a TRS-80 Computer, the K_m and V_{max} were within 10% of those obtained by graphical analysis.

Results

Radiolabeling of factor IX. A critical consideration in the development of the radiopeptide release assay for activated human Factor IX was that the procedure for radiolabeling the zymogen be gentle enough to maintain the functional activity of the protein while promoting sufficient incorporation of radionuclide to permit a sensitive assay of Factor IX activation. The radiolabeling technique utilized was based on the method of Van Lenten and Ashwell (19), in which terminal sialic acid residues of glycoproteins are oxidized with periodate and subsequently, reduced with tritiated sodium borohydride. However, we found that the functional activity of human Factor IX was significantly reduced

1. *Abbreviation used in this paper:* TBS, Tris-buffered saline.

after exposure to oxidizing conditions recommended for bovine Factor X (6) and bovine Factor IX (5). We have therefore developed a procedure for radiolabeling human Factor IX utilizing oxidation conditions sufficiently gentle to retain coagulant activity while assuring satisfactory incorporation of radionuclide.

To accomplish this objective, we varied the concentration of sodium periodate added to oxidize sialic acid residues in purified Factor IX as indicated in Fig. 2. After reduction with tritiated sodium borohydride and dialysis, as described in Methods and in the legend to Fig. 2, the samples were assayed for Factor IX coagulant activity and counted for incorporation of tritium. A progressive decrease in Factor IX activity was observed with increasing concentration of $NaIO_4$. Only 40% of control Factor IX activity remained in the sample treated with 4.5 mM $NaIO_4$, whereas >85% remained with 1.8 mM $NaIO_4$, at which ~70% of maximal incorporation of tritium was achieved (Fig. 2). Therefore, in all subsequent labelings of 45 μM Factor IX, we utilized 1.8 mM $NaIO_4$ and NaB_3H_4 with specific radioactivity of 72–83 Ci/mmol. This consistently resulted in retention of >90% of Factor IX coagulant activity and specific radioactivities of the subsequently gel-purified protein of 475,000–780,000 cpm/μg (0.85–1.4 atoms of ^3H/Factor IX molecule). The high specific radioactivity and coagulant activity of Factor IX provided assurance that release of tritium occurred from functionally active molecules, and the >98% TCA precipitability of ^3H in the zymogen allowed us to detect <1% release of TCA-soluble ^3H.

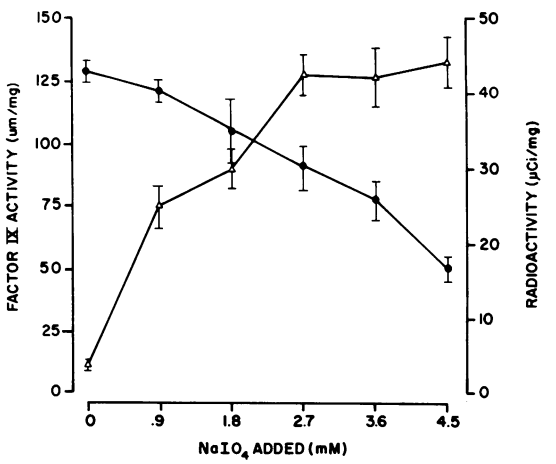

Figure 2. Effect of $NaIO_4$ concentration on ^3H-incorporation and coagulant activity of purified Factor IX. Partially purified Factor IX (eluate from sulfated dextran column prior to final purification by alkaline gel electrophoresis; see Methods), was equally divided into six aliquots of 125 μl each containing 0.51 mg protein. $NaIO_4$ was added to each sample as detailed in Methods to yield final concentrations as indicated in the figure. After quenching with ethylene glycol addition of ^3H-sodium borohydride and dialysis as detailed, each sample was counted in a scintillation counter and assayed for Factor IX coagulant activity.

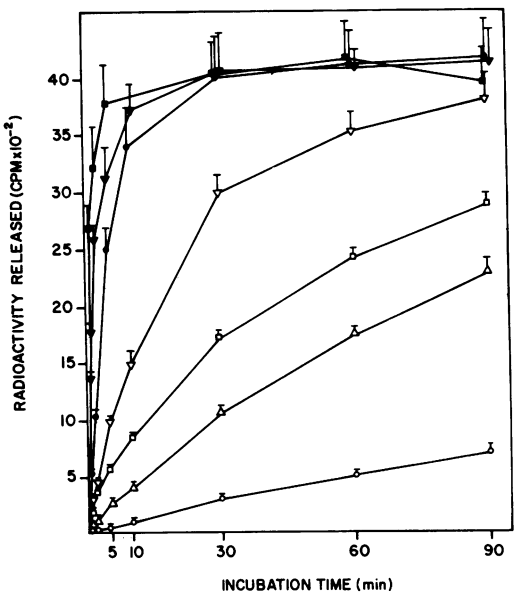

Figure 3. Release of TCA-soluble ^3H-labeled activation peptide from [sialyl-^3H] 35 nM Factor IX by various concentrations of Factor XIa (0.05–12.5 nM). Incubations were carried out as described in Methods, which also gives details of assay conditions. Each point represents the mean (±SEM) of triplicate determinations in two similar experiments carried out on two separate days. The concentrations of Factor XIa utilized were: 0.05 nM (○), 0.18 nM (△), 0.3 nM (□), 0.6 nM (▽), 3.1 nM (●), 6.2 nM (▼), and 12.5 nM (■).

Validation of assay for Factor IX activation. Time courses for the activation of Factor IX purified Factor XIa are shown in Fig. 3. The Factor XIa used in this experiment was prepared by incubating highly purified human Factor XI with bovine Factor XIIa as described in Methods. The concentrations of Factor XIa ranged from 0.008 to 2 μg/ml (0.05–12.5 nM) with a Factor IX concentration of 2 μg/ml (35 nM) giving molar ratios of Factor IX:Factor XIa of 2.8–700. In other similar experiments, the initial rates of TCA-soluble ^3H-release were linear over 10–30 min of incubation of Factor IX (5 μg/ml or 87.5 nM) with $CaCl_2$ (5 mM) and the Factor XIa at concentrations ranging from 0.01 to 0.2 μg/ml (0.062–1.25 nM). The progress curves shown for high enzyme concentrations (i.e., Factor IX:Factor XIa ratios > 12) in Fig. 3 and in all other experiments reached a plateau within 90 min when 40–42% of the total radioactivity was soluble in TCA, and this value was thereafter taken to represent 100% activation of Factor IX. This value is in reasonably good agreement with results obtained by extrapolating time courses to infinity by Zur and Nemerson (12) equating 38% release of TCA-soluble ^3H with 100% activation of Factor IX by Factor VIIa in the presence of tissue factor. Samples of [sialyl-^3H]Factor IX analyzed by SDS-gel electrophoresis in the presence of reducing agents before and after activation by Factor XIa showed >90% conversion of the zymogen Factor IX to cleavage products comprising the heavy

and light chains with apparent molecular weights of ~28,000 and 16,000 (Fig. 1, part *A*, lanes 1 and 2).

To determine whether the release of TCA-soluble ^{3}H-labeled activation peptide was correlated with the concomitant appearance of Factor IXa coagulant activity, the experiment shown in Fig. 4 was carried out as described in the legend. The release of ^{3}H-labeled activation peptide from 0.35 μM [^{3}H-sialyl]Factor IX by 0.625 nM Factor XIa (560:1 molar ratio) was followed by the development of Factor IXa activity after an apparent lag. This lag may reflect the possibility that the log-log transform used to calculate results of clotting assays minimizes but does not eliminate the inherent sigmoidicity of the clotting assay. In contrast, the radiometric assay is exquisitely sensitive and precise, and initial rates are linear. One alternative explanation, provided it can be confirmed more rigorously that the lag in development in coagulant activity is real, is that following the release of the activation peptide, a subsequent conformational alteration in Factor IX is required for development of coagulant activity. However, this latter explanation is highly speculative, and the major conclusion to be drawn from the experiment in Fig. 4 is that the release of activation peptide from Factor IX is either accompanied by or is shortly followed by the development of Factor-IXa coagulant activity. Therefore, activation peptide release would appear to be a reasonable measure of Factor IX activation by Factor XIa.

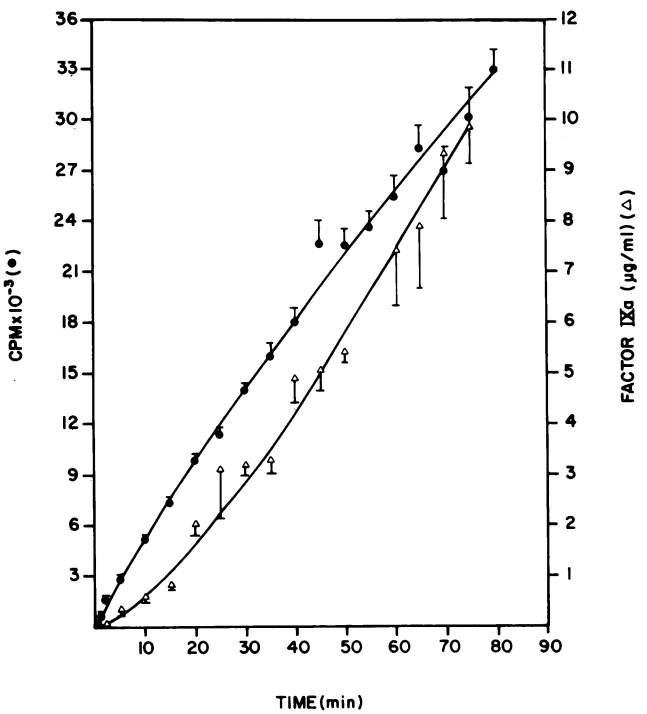

Figure 4. Time course of ^{3}H-labeled activation peptide released from [sialyl-^{3}H]Factor IX (0.35 μM or 20 μg/ml) by Factor XIa (6.2 nM or 0.1 μg/ml) and development of Factor IXa coagulant activity. The two proteins were incubated together in the presence of $CaCl_2$ (5 mM) in a reaction volume of 2 ml. At the indicated time intervals, 80 μl samples were removed into 270 μl of ice-cold EDTA (27 mM), Tris (67 mM), NaCl (133 mM), bovine serum albumin (1.33 mg/ml), pH 7.4, from which 40 μl was removed for determination of Factor IXa clotting activity as indicated in Methods. To the remaining 320-μl sample, 160 μl of 15% TCA was added for determination of acid soluble ^{3}H-release as detailed in Methods. Each point represents the mean±SEM of triplicate determinations of TCA ^{3}H release (○) or Factor IXa activity (△).

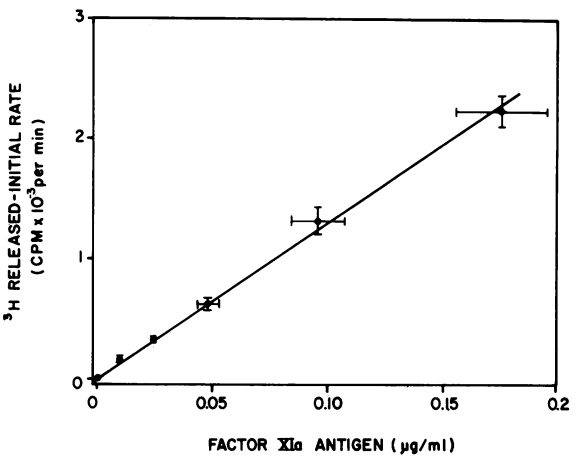

Figure 5. Relationship between activation peptide released from [sialyl-^{3}H]Factor IX and added amount of Factor XIa measured by radioimmunoassay. Factor IX (88 nM or 5 μg/ml) was incubated at 37°C with various concentrations of Factor XIa in the presence of 5 mM $CaCl_2$ in a final volume of 200 μl. At 5 and 10 min of incubation, 80 μl aliquots were removed into 240 μl of ice-cold EDTA (33 mM), Tris (16.7 mM), NaCl (33 mM), pH 7.5, for measurement of TCA-soluble tritiated peptide release as described in Methods. The various concentrations of Factor XIa used to activate Factor IX were assayed independently for Factor XI antigen by radioimmunoassay as described in Methods. The results represent the means (±SEM) of triplicate determinations.

To confirm the validity of the radiometric activation peptide release assay as a measure of Factor XIa activity, the experiment depicted in Fig. 5 was carried out. Factor IX was incubated with Factor XIa at Factor IX:Factor XIa molar ratios ranging between 70 and 5600 in the presence of $CaCl_2$ (5 mM). Independent measurements were made of initial rates of TCA-^{3}H-labeled activation peptide release and the amount of Factor XIa added. The Factor XIa added was measured independently by radioimmunoassay which was well correlated with measurements of Factor XIa functional activity by coagulation and chromogenic assays (see Methods and Reference 22). The results demonstrate a linear correlation ($r > 0.98$, $P < 0.001$) between added Factor XIa and activation peptide released, which validates the radiometric assay as a measure of Factor XIa enzymatic activity.

Kinetics of Factor IX activation by Factor XIa. To determine kinetic parameters for the activation of Factor IX by Factor XIa, varying concentrations of ^{3}H-labeled Factor IX were in-

cubated with Factor-XIIa-activated Factor XIa at Factor IX:Factor XIa molar ratios of 140–7,000. Initial rates of Factor IX activation were determined, utilizing the activation peptide release assay. These initial rates were determined over a 10 min incubation period during which 0.5–5% of the substrate was hydrolyzed (depending on the substrate concentration), and the rates of hydrolysis were linear. Initial rates were determined by calculating rates at each of five separate time points and averaging them. The results of a representative experiment are plotted in Fig. 6 as reciprocal initial rates vs. reciprocal substrate concentrations. Kinetic parameters obtained from these results were: K_m = 0.25 μM; V_{max} = 1.25 nmol/liter per s; k_{cat} = 10/s; k_{cat}/K_m = 40/s·μM. The experiment shown in Fig. 6 was carried out with varying amounts of a mixture of four parts of unlabeled Factor IX and one part of ^{3}H-labeled Factor IX. When the experiment was repeated with varying amounts of tritiated Factor IX without cold Factor IX added, virtually identical results were obtained. Therefore, it would appear that tritiation of Factor IX does not affect it as a substrate for Factor XIa. Summary data from four separate similar experiments are shown in Table I.

Discussion

The objective of this study was to examine the kinetics of activation of human Factor IX by human Factor XIa in order to provide information to help assess the physiological relevance of contact activation in the initiation of intrinsic coagulation.

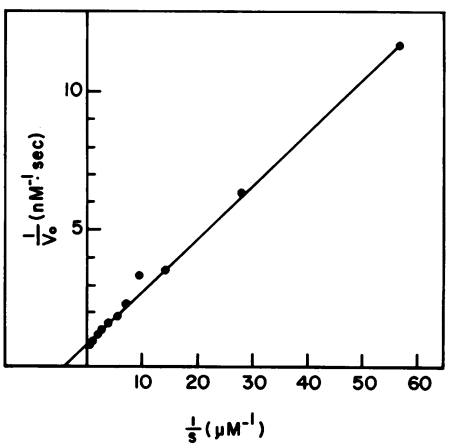

Figure 6. Lineweaver-Burk plot of the activation of Factor IX by Factor XIa. Mixtures of four parts cold Factor IX to one part [sialyl-^{3}H]Factor IX, at final concentrations of 0.0175–0.877 μM, were incubated at 37°C (500 μl reaction volumes) with 0.125 nM Factor XIa and 5 mM $CaCl_2$ in 50 mM Tris, 100 mM NaCl, 1 mg/ml bovine serum albumin, pH 7.5. At 0, 1, 2, 5, and 10 min incubation times, 80-μl samples were withdrawn into 240 μl of ice-cold EDTA (33 mM), Tris (16.7 mM), NaCl (33 mM), pH 7.5, for measurement of TCA-soluble tritiated peptide release as described in Methods.

*Table I. Steady State Kinetic Parameters for Factor IX Activation by Factor XIa**

K_m	V_{max}	k_{cat}	k_{cat}/K_m
μM	nmol/l·s	s^{-1}	s^{-1} μM^{-1}
0.49±0.16	0.96±0.21	7.70±1.68	16.47±2.29

* Data represent mean±SD for four similar separate experiments. Conditions were those detailed in the legend to Fig. 6.

Therefore, an assay for Factor-IX activation was required. Conventional coagulation assays lack specificity, whereas suitable small peptide chromogenic substrates are not available for Factor IXa. The tritiated activation peptide release assay developed in our laboratory for human Factor IX activation was based on similar assays for the activation of bovine Factor X (6) and bovine Factor IX (5). It takes advantage of the distribution of carbohydrate in the molecule, 50% of which resides in the 35 amino acid activation peptide that is released when the single chain zymogen is cleaved by Factor XIa at Arg^{145}-Ala^{146} and at Arg^{180}-Val^{181} (9, 31). The assay is therefore highly specific for Factor IX activation since it monitors a physical property of the molecule which correlates well with the development of coagulant activity (Fig. 4) and with the enzymatic activity of added Factor XIa (Fig. 5), which was measured by chromogenic, coagulation, and radioimmunologic assays (22). In addition, the assay is highly sensitive owing to low background since more than 98% of the radioactivity of the zymogen is precipitated in 5% TCA, permitting the detection of 1% activation of Factor IX. The incorporation of 0.85–1.4 atoms of ^{3}H/Factor IX molecule provides assurance that those substrate molecules from which tritium is released are functionally active, and confirmation of this supposition comes from experiments (see Results) in which identical percentages of peptide release were obtained when the ratio of unlabeled to [^{3}H]-labeled Factor IX was varied. Thus, the radiopeptide release assay provides a specific, sensitive, and reliable method for examining the kinetics of Factor IX activation by Factor XIa.

The kinetic parameters obtained in our laboratory for Factor IX activation by Factor XIa differ considerably from those obtained by Bajaj (32), who found a higher K_m (2.0 μM, compared with 0.49 μM, Table I), and a lower k_{cat} (0.17/s, compared with 7.7/s, Table I). The catalyst used in the study reported by Bajaj (32) was trypsin-activated Factor XIa, whereas we have employed Factor XI activated by bovine Factor XIIa. We began our studies with Factor XI incubated with insolubilized trypsin suspension. When the Factor XI was examined by reduced SDS-gel electrophoresis during the course of activation, we observed the appearance of proteolytic degradation products with molecular weights lower than the heavy and light chains of Factor XIa even before the disappearance of the 80,000 M_r zymogen. Furthermore, the concentrations of trypsin-activated Factor XIa required for comparable rates of Factor IX activation were

10–100-fold higher than those of Factor XIIa-activated Factor XIa. However, a direct and rigorous comparison of the two Factor XIa preparations was not made, and the studies reported here were done exclusively with the Factor XIIa-activated enzyme.

The ultimate aim of this study is to acquire information useful in assessing the contribution of contact activation to the activation of Factor IX. The K_m derived here for the Factor XIa catalyzed reaction, 0.49 μM (Table I), is approximately five- to sixfold higher than the plasma concentration of Factor IX (~88 nM or 5 μg/ml), as estimated from the specific activity of purified Factor IX (4) and by immunological techniques (3). The concentration of Factor IX in plasma could therefore be expected to regulate the rate of the reaction. The k_{cat} obtained in the present study was 7.7/s (Table I). The k_{cat} obtained by Zur and Nemerson (12) for the Factor VIIa-tissue factor catalyzed activation of Factor IX was dependent upon the tissue factor concentration and varied from 0.15 to 0.42/s depending on thromboplastin concentration. We recognize that direct comparisons of kinetic parameters between laboratories are difficult to interpret since experimental conditions and materials vary and the source of proteins differs (bovine or human). Furthermore, the relative rate of Factor IX activation by Factor XIa (or by Factor VIIa) depends upon a number of factors, including the concentration of Factor XIa (or Factor VIIa) generated in vivo, as well as the presence of possible cofactors which might influence the kinetic parameters. In the case of the Factor VIIa-catalyzed reaction, thromboplastin is an obligatory cofactor (10). K_m values as well as V_{max} values increase as a consequence of increasing tissue factor concentration, so that the net effect is a slight increase in catalytic efficiency at an optimal thromboplastin concentration (12). Thus, the kinetic parameters for the extrinsic activation of Factor IX are condition-dependent and can not be simply related to a one enzyme, one substrate reaction such as the Factor XIa catalyzed reaction. A definitive evaluation of the relative efficiencies of these two reactions will require a great deal of experimental and mathematical work.

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