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Activation of Factor IX by Factor XI_a – A Spectrophotometric Assay for Factor IX in Human Plasma*

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Key words

Active site titration of F IX_a – Activation of F IX by F XI_a – Spectrophotometric assay for F IX_a – Chromogenic substrate – Factor XI_a – Factor IX – Hemophilia B

Summary

The activation of Factor IX by partially purified Factor XI_a was followed by active site titration, gelelectrophoresis and by a spectrophotometric assay. The assay is based on the finding that the rate of Factor X activation in the presence of phospholipid and Ca^{2+} is linear in time and proportional to the amount of Factor IX_a present and can be determined with the chromogenic substrate S2222. Conditions were found that allowed complete activation of Factor IX in human plasma by Factor XI_a. The amount of Factor IX_a present in the plasma sample can be determined with the spectrophotometric assay and is proportional with the amount of plasma present. In plasma from patients receiving vitamin-K antagonists reduced Factor IX activity is found with the spectrophotometric assay, and the new assay method may be useful in monitoring oral anticoagulant therapy.

Introduction

Factor IX (Christmas Factor, Antihemophilia Factor B)[1] is the zymogen of the serine protease Factor IX_a. Factor IX_a participates in the intrinsic pathway of blood coagulation as the enzyme that activates Factor X, in a reaction accelerated by the nonenzymatic cofactors, Factor $VIII_a$, and phospholipids plus calcium ions (1, see ref. 2 and 3 for a review).

Bovine and human Factor IX have been purified and characterized, and were found very similar (4–9). Bovine Factor IX is a glycoprotein with a molecular weight of 55,400 containing a single polypeptide chain of known amino acid sequence (10). Factor $IX_{a\beta}$ with a molecular weight of 45,000 consists of two polypeptide chains held together by disulfide bonds. Factor IX is converted to Factor $IX_{a\beta}$ by Factor XI_a in the presence of calcium ions. During activation two internal peptide bonds Arg^{146}-Ala^{147} and Arg^{181}-Val^{182} are cleaved and a carbohydrate rich activation fragment of molecular weight 10,000 is released (11, 12; see Fig. 1).

The complex of tissue factor and factor VII_a also activates Factor IX, possibly by cleavage of the same two bonds (13, 14).

The Factor X activating protease from Russell's viper venom (RVV-X)[2], converts Factor IX to Factor $IX_{a\alpha}$, by cleavage of only the internal Arg^{181}-Val^{182} bond, without release of the activation fragment (12). The calcium requirement and the role of other metal ions in Factor IX activation by Factor XI_a and RVV-X has been investigated (15).

Several methods are used to determine Factor IX and to follow its activation to Factor IX_a. These methods are (1–4): 1. A clotting assay using Factor IX deficient patient plasma (16). 2. A method based on the esterase activity of Factor IX_a using radiolabelled esters, like benzoyl-L-arginine [3H] ethyl ester (17). 3. Method of Nemerson based on the finding that Factor IX is acid precipitable but its carbohydrate rich activation fragment is acid soluble. Thus when a Factor IX preparation that is radiolabelled in the carbohydrate rich fragment is activated, its conversion to Factor IX_a can be followed by determination of the release of acid soluble radioactivity (14, 18). 4. Active site titration. Byrne et al. determined the concentration of Factor IX_a by active site titration with pNPGB[2], and reported the kinetic constants for the reaction between Factor IX_a and pNPGB (17). The methods 2, 3 and 4 can only be used with purified Factor IX or IX_a, whereas method 4 requires large amounts of Factor IX_a. Only method 1, the clotting assay can be used to determine Factor IX in plasma.

Our recent work on intrinsic Factor X activation suggested that a spectrophotometric assay may be feasible. Therefore in the present study we used the technique of active site titration to obtain a Factor IX_a preparation of known molar concentration and devised a spectrophotometric assay for purified Factor IX_a and calibrated it with active site titrated Factor IX_a. The spectrophotometric assay was used to study the activation of plasma Factor IX by Factor XI_a, in order to develop a new method for the determination of Factor IX in human plasma.

Materials and Methods

Materials. S2222[2] was purchased from AB Kabi Diagnostica, Stockholm, Sweden; pNPGB was from Nutritional Biochemicals; Russell's Viper Venom, STI[2], egg-yolk phosphatidylcholine and ovalbumin were obtained from Sigma. DEAE-Sephadex A50, Sephadex G25, G100 and Sepharose 4 B were from Pharmacia. Heparin (unbleached) used to make heparin agarose (4, 20) was donated by Dr. G. van Dedem, Diosynth B.V. Oss, The Netherlands. All reagents used were of the highest grade commercially available.

Plasma. Human reference plasma was citrated platelet poor plasma obtained from thirty healthy male and female donors. Factor IX and VIII

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[1] The nomenclature of the blood coagulation factors used is that recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and zymogen Intermediates.

[2] The abbreviations used are: pNPGB, p-nitrophenyl-p-guanidinobenzoate hydrochloride; S2222, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride; RVV-X, purified Factor X activator from Russell's viper venom; STI, soybean trypsin inhibitor.

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deficient plasma was obtained from local patients; Factor VII deficient plasma was from Merz and Dade, Switzerland.

Coumarin plasma was citrated platelet poor plasma pooled from patients receiving long term anticoagulant therapy (Marcoumar) and was provided by Dr. G. Kok, St. Annadal Hospital, Maastricht.

Proteins. Bovine Factor IX was purified as described by Fujikawa et al. (4). Bovine Factor X_1 and X_2 were prepared according to Fujikawa et al. (21). The Factor IX, X_1 and X_2 preparations were homogenous and pure as determined by gelelectrophoresis in the presence of sodium dodecyl sulfate. The specific activities of these preparations, as determined with a clotting assay, were equal to those reported (4, 21). Factor XI_a, used in this study, was partially pure and isolated from bovine contact product, made according to Nossel (22) and further purified by heparin-agarose affinity chromatography as suggested by Østerud et al. (13). Contact product was applied to the heparin-agarose column (1.5 × 15 cm) in a buffer containing 0.05 M sodium acetate and 0.3 M NaCl at pH 5.5 and was eluted with a linear gradient of 0.3 to 1.0 M NaCl in 0.05 M sodiumacetate at pH 5.5. RVV-X was purified from the crude venom as described by Schiffman et al. (23). Bovine Factor X_a was prepared from bovine Factor X_2 using RVV-X according to Fujikawa et al. (24). Bovine Factor IX_a was prepared by incubating Factor IX (2 mg/ml) at 37° C with Factor XI_a (38 µg/ml) in a buffer containing 50 mM Tris-HCl, 50 mM NaCl at pH 8.5 in the presence of 10 mM $CaCl_2$ (11). After 60 min incubation the reaction mixture was brought to 15 mM EDTA and 20 mM benzamidine and applied to a column of DEAE-Sephadex A50 (1.5 × 30 cm) in 50 mM Tris-HCl, 50 mM NaCl and 20 mM benzamidine at pH 7.9. Factor IX_a was eluted with a linear gradient of 50 mM to 400 mM NaCl (2 × 500 ml) in 50 mM Tris-HCl, 20 mM benzamidine at pH 7.9.

Phospholipids and phospholipid vesicle preparation. Brain phosphatidylserine was prepared as described by Sanders (25). Single bilayer vesicle solutions of a mixture of brain phosphatidylserine and egg-yolk phosphatidylcholine (25/75 w/w) were prepared according to de Kruijff et al. (26) by sonication for 10 min in 50 mM Tris-HCl, 175 mM NaCl at pH 7.9. Sonication was performed using a MSE Mark II 150 Watt ultrasonic disintegrator set at 10 microns peak to peak amplitude. After sonication no pH adjustment was needed.

Active site titration of Factor IX_a. Titration experiments were conducted at 37° C in thermostated cuvettes in an Aminco DW-2a spectrophotometer set in the split beam mode at 405 nm. In a typical experiment the sample cuvette contained 40 µg Factor IX_a in 800 µl 0.05 M sodium veronal buffer (pH 8.3) in the presence of 20 mM $CaCl_2$. The reference cuvette contained 800 µl 0.05 M sodium veronal buffer and 20 mM $CaCl_2$. After an appropriate time to allow for temperature equilibration 5 µl of a 0.02 M solution of pNPGB in dimethylformamide was simultaneously added to the sample and the reference cuvette using matched micropipettes. Under these conditions the presteady state part of the reaction is completed in about 6 min. When Factor IX at comparable concentrations is allowed to react with pNPGB no burst of p-nitrophenol production is observed. However, Factor IX gives a low steady state production of p-nitrophenol.

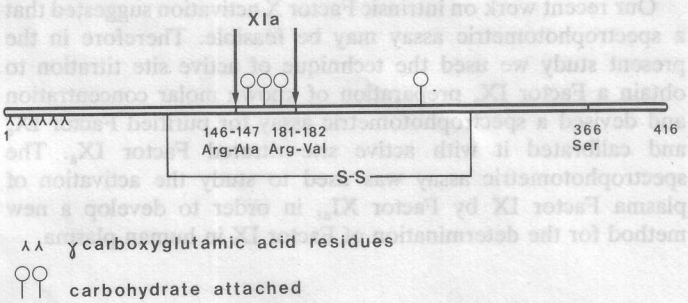

Fig. 1 Diagram of bovine factor IX. Factor IX is composed of a single polypeptide chain of 416 aminoacids. The carboxyterminal part of the molecule is homologous with trypsin, with the active site serine residue at position 366. Twelve γ-carboxyglumatic acid residues are located among the first 40 aminoacids from the N-terminus. Four asparaginyl residues have carbohydrate attached with total molecular weight of 8,100. Activation by Factor XI_a, is indicated by arrows, and involves cleavage between Arg^{146}-Ala^{147} residues and between Arg^{181}-Val^{182}. The peptide of 34 aminoacids containing most of the carbohydrate is released giving rise to Factor $IX_{a\beta}$. The light and heavy chains are connected by S-S bond(s). RVV-X activation results in Factor $IX_{a\alpha}$, with only the Arg^{181}-Val^{182} bond cleaved (Data from Katayama et al., 10).

Theory of titration. When a serine esterase is active site titrated with pNPGB, a rapid burst of p-nitrophenol is observed followed by a very slow or negligible steady state production of p-nitrophenol. Bender et al. (27, 28) have derived kinetic equations for this process according to a three-step mechanism.

$$E + pNPGB \overset{K_s}{\rightleftharpoons} E.NPGB \overset{k_2}{\rightarrow} E-GB + NP \overset{k_3}{\rightarrow} E + GB.$$

Here E is the enzyme, pNPGB is the substrate, E.NPGB is the enzymesubstrate complex, E−GB is the guanidinobenzoyl enzyme that results from the stoichiometric reaction of enzyme with substrate liberating 1 eq of p-nitrophenol (NP) and GB is the free p-guanidinobenzoate that results from the deacylation of the E−GB complex.

Factor IX_a was reacted with various concentrations of pNPGB and the appearance of p-nitrophenol in time recorded. The data were treated according to Bender et al. (27, 28) to obtain the various parameters. The deacylation rate constant k_3 was determined according to Chase and Shaw (29) by isolation of the acylenzyme intermediate and following the reappearance of enzyme activity in time with the spectrophotometric assay (see below). Because of the similarity in methods and results we present the final results together with those from ref. 19 in Table 1. An extensive account of the method can be found in ref. 27, 28, 19.

Spectrophotometric assay for Factor IX_a. Factor IX_a converts Factor X to Factor X_a in the presence of negatively charged phospholipids and calcium ions. Conditions can be chosen such that the rate of Factor X_a formation is constant in time and proportional to the amount of Factor IX_a present in the reaction-mixture (1; see also Results Section). Sonicated phospholipid vesicles 0.5 mM, in 50 mM Tris, 175 mM NaCl pH 7.9 were brought to 50 mM $CaCl_2$ and incubated for 10 min at 37° C. 0.1 ml of the vesicle-calcium suspension is added to 0.3 ml of a mixture containing 0.5 nmol of Factor X, 50 mM Tris-HCl pH 7.9, 175 mM NaCl and 1 mg/ml ovalbumin. This mixture was incubated for 4 min at 37° C and Factor X activation was started by addition of 0.1 ml of the Factor IX_a sample to be analysed. After 10 min at 37° C, Factor X_a formation was stopped by transferring 0.4 ml to the cuvette with 1.6 ml of a buffer containing 50 mM Tris-HCl pH 7.9, 175 mM NaCl, 20 mM EDTA, 241 µM S2222 and 0.5 mg/ml ovalbumin. From the absorbance change at 405 minus 500 nm measured on an Aminco DW-2a spectrophotometer in the dual wavelength mode and a calibration curve, made by measuring the rate of S2222 hydrolysis with known amounts of active site titrated Factor X_a (30), the amount of Factor X_a present in the reaction mixture can be calculated.

Gelelectrophoretic analysis of Factor IX activation. Gelelectrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (31) with gels containing 10% acrylamide, 0.27% N,N^3-methylenebisacrylamide and 0.1% sodium dodecylsulfate. To follow the activation of Factor IX by Factor XI_a or by RVV-X, aliquots of the activation mixture (10 µl) were diluted 30 fold in 2% sodium dodecylsulfate and were kept for 3 min in a boiling waterbath. 5% Mercaptoethanol was present in disulfide reduced samples. 50 µl of each sample was applied to a gel. After electrophoresis, gels were removed from the tubes, and stained and destained according to Fairbanks (32). Finally, the gels were scanned on a Gilford Model 250 spectrophotometer.

The amount of protein present in the gel bands was estimated with calibration curves obtained by running known amounts of Factor IX and Factor IX_a on unreduced and reduced gels. After staining, destaining, scanning and determining the surface area of the peakscans, calibration curves were obtained that were used to quantitate Factor IX, the activation intermediate and Factor IX_a present in the gels (reduced and unreduced) that were run to follow Factor IX activation.

Results

Active Site Titration of Factor IX_a

Ideal active site titrants for proteolytic enzymes are compounds that bind to and are rapidly cleaved by the enzyme. After

cleavage, one part of the titrant remains bound to the enzyme thereby blocking the active site whereas the other part usually carrying a chromophoric group like p-nitrophenol is released and can be measured spectrophotometrically. Quantitation of the enzyme is simple since from the amount of chromophore released, the molar concentration of enzyme can be calculated (27).

The compound pNPGB has been used for active site titration of trypsin and the trypsinlike enzymes, thrombin and Factor X_a (27, 30). Its usefulness for Factor IX_a was assessed by determination of the kinetic parameters for the reaction. After completion of this work Byrne et al. (19) reported the kinetic parameters, determined under slightly different reaction conditions. Therefore in Table 1, we only show our final results, together with the data of Byrne et al. (19). The results are in good agreement and justify the use of pNPGB as an active site titrant for Factor IX_a.

Different preparations of Factor IX_a were active site titrated with pNPGB and dependent on the preparation 0.70–0.95 active sites/mole of Factor IX_a were found. The molar concentration of Factor IX_a was calculated from the absorbance at 280 nm using $A^{1\%}_{1cm} = 14.3$ (11) and a molecular weight of 46,000. For Factor IX a molecular weight of 56,000 and a $A^{1\%}_{1cm} = 14.9$ was used (11).

When Factor IX was activated with either Factor XI_a or RVV-X and active site titration was carried out directly with a sample from the activation mixture, 0.95–1.00 active sites/mole of Factor IX were generated. Apparently some loss of active sites may occur during further IX_a purfication. The results also indicate that cleavage of Factor IX by Factor XI_a or by RVV-X results in the formation of one active site per molecule of zymogen.

Spectrophotometric Assay for Factor IX_a

No specific chromogenic substrate for Factor IX_a is presently available. However, in a recent study of the kinetics of Factor X activation by Factor IX_a we have shown that the rates of Factor X_a formation in the presence of phospholipids and calcium ions but without Factor $VIII_a$ were sufficiently high to be measured with the chromogenic substrate S2222 (1). Under these conditions, the rate of Factor X_a formation is linear in time and as shown in Fig. 2, the rate is proportional to the amount of Factor IX_a, at least up to 4 µg/ml of Factor IX_a.

The assay was not influenced by the presence of Factor XI_a. Factor XI_a in concentrations as used in the assay did not activate Factor X and Factors IX_a and XI_a did not hydrolyse S2222 directly.

Activation of Bovine Factor IX by Factor XI_a

Bovine Factor IX was incubated with Factor XI_a in the presence of calcium ions (11). At various times samples were taken and the molecular changes studied with polyacrylamide gel electrophoresis with and without reduction (Fig. 3 A, B). During activation an intermediate appears rapidly, that is converted more slowly to the end product. From the work of Lindquist et al. (12) it can be concluded that the intermediate represents Factor IX cleaved at the Arg^{146}-Ala^{147} site, which is later cleaved at Arg^{181}-Val^{182} to give Factor $IX_{a\beta}$. The amounts of protein present in the various bands on the gels were estimated as described in the Materials and Methods section, and are shown in Fig. 3 C. Reaction samples were further examined for presence of active sites titratable with pNPGB and for Factor IX_a activity determined in the spectrophotometric assay. The results together with those from the gels are shown in Fig. 3 D. The appearance of double cleaved Factor IX_a on gels; of active sites titratable with pNPGB and of enzymatic activity towards Factor X is synchronous. Therefore, appearance of the active site and of enzyme activity are associated with the Arg-Val cleavage and no activity is

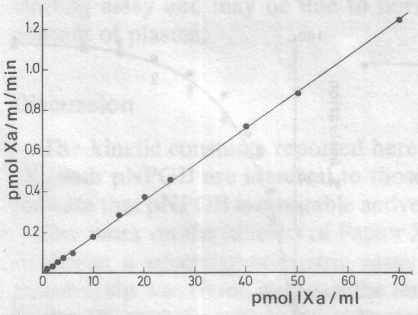

Fig. 2

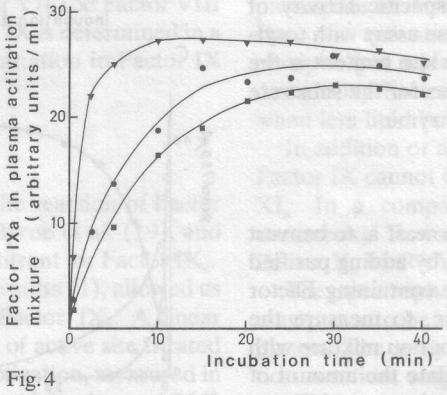

Fig. 4

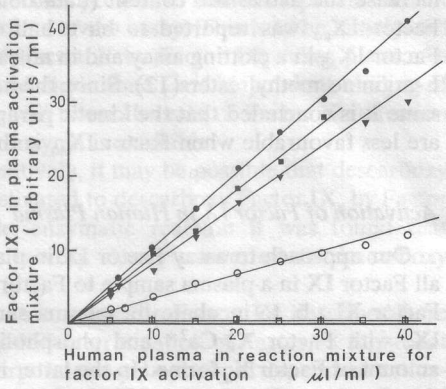

Fig. 5

Fig. 2 Calibration curve of the rate of factor X_a formation with varying amounts of active site titrated bovine Factor IX_a. Factor IX_a was incubated with purified Factor X, phospholipid vesicles and Ca^{2+} and the amount of Factor X_a formed after 10 min was determined with the chromogenic substrate S2222. Conditions were as described in Materials and Methods.

Fig. 4 Time course of Factor IX activation in human plasma by Factor XI_a. Human reference plasma, 20 µl, was incubated with varying amounts of partially purified Factor XI_a in a reaction mixture of 1 ml containing 50 mM Tris-HCl, pH 7.9, 175 mM NaCl, 10 mM $CaCl_2$ and 0.5 mg/ml ovalbumin. After the time intervals indicated in the figure samples of 0.1 ml were added to 0.4 ml of a Factor X activation mixture containing Factor X, Ca^{2+} and phospholipids as described in the Methods section and the amount of Factor X_a present after 10 min was calculated as in Fig. 2. Using the calibration curve of Fig. 2, the amount of bovine Factor IX_a that would produce this amount of Factor X_a was calculated. From this amount the amount of factor IX_a present in the plasma Factor IX activation mixture was calculated. Thus, one arbitrary unit of plasma Factor IX_a, as indicated at the ordinate, is equal to the activity of 1 pmole of purified bovine Factor IX_a in the spectrophotometric assay. The concentrations of Factor XI_a used were: ▲—▲ 3 µg/ml, ●—● 1.5 µg/ml, ■—■ 0.75 µg/ml.

Fig. 5 Calibration curves of Factor IX in human reference plasma, in coumarin plasma and in plasma from patients congenitally deficient in Factor VII, VIII or IX. Varying amounts indicated in the figure of different human plasmas were activated with 1.5 µg Factor XI_a in a final reaction volume of 1 ml containing 50 mM Tris-HCl pH 7.9, 175 mM NaCl, 10 mM $CaCl_2$ and 0.5 mg/ml ovalbumin. After 25 min incubation at 37° C, a sample was taken and the amount of Factor IX_a present was determined as described in the legend of Fig. 4. ●—● Human reference plasma, ■—■ Factor VII deficient plasma, ▲—▲ Factor VIII deficient plasma, □—□ Factor IX deficient plasma, ○—○ Coumarin plasma.

Table 1 Kinetic constants for the reaction of bovine Factor IX_a with pNPGB. Active site titration of bovine Factor IX_a was carried out as described in Materials and Methods. The calculation of kinetic parameters and rate constants is based on the titration theory of Bender et al. (27, 28).

Kinetic parameters	This work 20 mM Ca^{2+} 37° C	Byrne et al. (17) 10 mM Ca^{2+} 30° C
K_s (μM)	187	520
k_2 (5^{-1})	0.056	0.13
k_3 ($10^5 \times 5^{-1}$)	3	1
k_2/k_3	1,875	13,000
K_m app (μM)	0.1	0.04

Table 2 Comparison of clotting and spectrophotometric assays for Factor IX in human plasmas. The data obtained with the spectrophotometric assay were calculated from the results shown in Fig. 5. The Factor IX content of the same plasmas was determined in a one-stage clotting assay using Factor IX deficient plasma (16).

Plasma	Clotting assay	Spectrophotometric assay
Normal reference	100%	100%
Factor IX deficient	< 2%	< 1%
Factor VII deficient	65%	88%
Factor VIII deficient	76%	80%
Coumarin	26%	31%

available in the intermediate. This is in agreement with the finding that the intermediate lacks coagulant and esterase activity (12).

When Factor IX is converted by RVV-X to Factor $IX_{a\alpha}$, the same number of active sites were found (19). Subsequent conversion of Factor $IX_{a\alpha}$ to Factor $IX_{a\beta}$ with Factor XI_a did not increase the active site content (data not shown). Nevertheless, Factor $IX_{a\alpha}$ was reported to have half the specific activity of Factor $IX_{a\beta}$ in a clotting assay and in an esterase assay with tosyl-L-arginine methyl ester (12). Since the active site content is the same it is concluded that the kinetic parameters for the substrate are less favourable when Factor $IX_{a\alpha}$ is the enzyme.

Activation of Factor IX in Human Plasma

Our approach to assay Factor IX in plasma was: a. to convert all Factor IX in a plasma sample to Factor IX_a by adding purified Factor XI_a; b. to incubate the plasma sample containing Factor IX_a with Factor X, Ca^{2+} and phospholipid c. to measure the amount of Factor X_a formed in the latter incubation mixture with the chromogenic substrate S2222; d. to calculate the amount of Factor IX_a present in the plasma sample from the rate of Factor X_a formation and a calibration curve made with known amounts of Factor IX_a (see Fig. 2).

In Fig. 4 time courses are shown for the generation of Factor IX_a in human plasma. When 20 μl of plasma is incubated at 10 mM Ca^{2+} and 0.75 μg of Factor XI_a in a reaction volume of 1 ml, a plateau level of Factor IX_a activity is reached after 30 min (Fig. 4, squares). At twofold, respectively fourfold higher Factor XI_a concentrations the plateau level remains approximately the same, but is reached after 20, respectively 10 min of activation. Using these reaction conditions, varying amounts of plasma (5–40 μl) were incubated for 25 min with 1.5 μg/ml Factor XI_a. The results (Fig. 5, closed circles) indicate that the Factor IX_a activity that is generated is proportional to the amount of plasma. From these experiments we conclude that under our reaction conditions all Factor IX in plasma can be converted to Factor IX_a.

When the amount of plasma present in the reaction mixture for Factor IX activation was increased from 40 till 100 μl plasma, the increase in Factor IX_a activity was slightly less than proportional and with 200–500 μl per ml only a small increase in Factor IX_a activity was found. This phenomenon may be due to inactivation or inhibition of Factor XI_a or Factor IX_a and was not further investigated.

Therefore a standard Factor IX determination in plasma is carried out as described in the legend of Fig. 5 with 20 μl of plasma per ml reaction mixture. This will result in an absorbance change of 0.0081 Δ A405/min. For spectrophotometers less sensitive than our Aminco DW 2a, higher absorbance changes may be required. These can be obtained by prolonging the Factor X activation step or by transfer of larger amounts of the Factor X_a reaction mixture to the cuvette.

A major condition for the assay is absence of interference by other plasma components. Therefore several control experiments were done to verify that the Factor X activating activity that

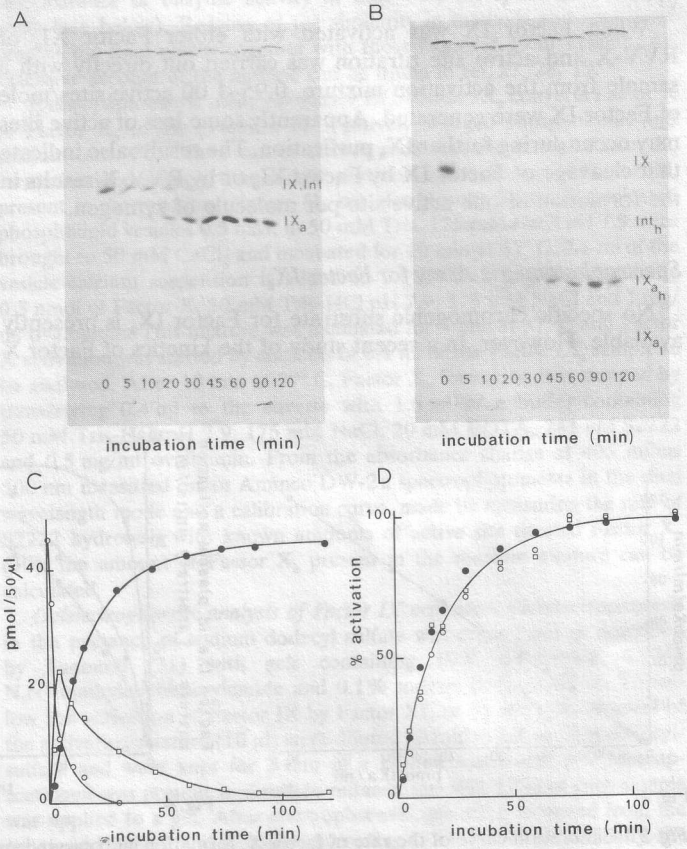

Fig. 3 Activation of Factor IX by Factor XI_a, as followed by gelelectrophoresis, active site titration and by a spectrophotometric assay for Factor IX_a. Factor IX (1.5 mg/ml) was activated with partially purified Factor XI_a (30 μg/ml) in a buffer containing 50 mM Tris-HCl, 50 mM NaCl pH 8.5, 37° C. After the time intervals indicated, samples were taken and active site titrated, assayed for Factor IX_a and applied to the gels. The gels were run, stained, destained, scanned and the protein present in the bands quantitated as described in the Methods section. (A) Gel pattern of Factor IX activation, without reduction; (B) as (A), with reduction, (Int, denotes the Factor IX activation intermediate, subscripts h and l, heavy and light chain, cf. Fig. 1); (C) Data obtained from gel scanning: ●—●, amount of Factor IX_a present in the sample; ○—○, amount of Factor IX in sample; □—□, amount of intermediate present in sample. (D) Time course of Factor IX activation determined by: ●—●, gel scanning; □—□, active site titration; ○—○, spectrophotometric assay for factor IX_a.

appears in plasma during incubation with Factor XI_a and Ca^{2+}, is due only to Factor IX_a.

By omitting Factor X from the assay it was found that during Factor IX activation in plasma no significant plasma Factor X activation occurs, since the amount of Factor X_a formed is negligible compared to the amounts of Factor X_a formed by Factor IX_a in the Factor X activation reaction. Therefore any Factor X_a that may have formed during plasma activation did not interfere with our spectrophotometric assay.

Fig. 5 (open squares) shows that in Factor IX deficient plasma no Factor IX_a, nor other Factor X activators are formed. Apparently Factor $VII_{(a)}$, another Factor X activator does not contribute to Factor X activation in our assay. Indeed (closed squares) Factor VII deficient plasma yields nearly the same level (88%) of Factor X activating activity (Factor IX_a) as human reference plasma.

Factor $VIII_a$ increases the V_{max} of Factor X_a formation by Factor IX_a (1). To rule out Factor $VIII_a$ involvement, Factor VIII deficient plasma was activated and found to contain 80% of the Factor IX in normal pooled reference plasma (triangles). When reference pool plasma was first clotted with thrombin, a treatment known to destroy Factor $VIII_a$ activity, and then activated with Factor XI_a and Ca^{2+}, the amount of Factor IX_a formed in our assay was equal to that found in the original plasma. Therefore under our conditions, the spectrophotometric assay is not influenced by Factor $VIII_a$.

In Fig. 5, it is further shown that plasma pooled from patients receiving coumarin, contains 31% of Factor IX in reference plasma when measured with the spectrophotometric assay (open circles). This suggests that the spectrophotometric assay like a clotting assay discriminates between normal Factor IX and the abnormal (descarboxy) Factor IX molecules that are present in coumarin plasma (compare Table 2).

Table 2 shows the Factor IX content of various plasmas determined with the spectrophotometric assay (Fig. 5) and with the coagulation assay using Factor IX deficient plasma. The slightly lower Factor IX content of the Factor VII and Factor VIII deficient plasmas, also appears when Factor IX is determined in a clotting assay and may be due to normal variation in Factor IX content of plasma.

Discussion

The kinetic constants reported here for the reaction of Factor IX_a with pNPGB are identical to those of Byrne et al. (19), and indicate that pNPGB is a suitable active site titrant for Factor IX_a.

Our work on the kinetics of Factor X activation (1), allowed us to devise a spectrophotometric assay for Factor IX_a. A linear relationship was found between the amount of active site titrated Factor IX_a and the initial rate of Factor X activation, measured in the presence of phospholipids (25% phosphatidylserine and 75% phosphatidylcholine) and calcium ions. The conversion of Factor IX to Factor $IX_{a\beta}$ with Factor XI_a and Ca^{2+} was followed with the spectrophotometric assay, with active site titration and with polyacrylamide gel electrophoresis. The results confirm the cleavage pattern of Factor IX and the accumulation of the intermediate as first found by Lindquist et al. (12). The intermediate without coagulant or esterase activity is also unreactive towards pNPGB. This is not simply due to presence of the activation fragment *per se* because RVV-X cleaved Factor $IX_{a\alpha}$ is an active enzyme. Cleavage at Arg^{181}-Val^{182} is simultaneous with appearance of active sites towards pNPGB and enzymatic activity towards Factor X. Apparently, cleavage of the Arg-Val bond is essential in formation of the active site.

Factor $IX_{a\alpha}$ and Factor $IX_{a\beta}$ have the same number of active sites (19) but Factor $IX_{a\alpha}$ has twice the clotting activity of Factor IX_a (12). The kinetic parameters of the two enzymes for ester hydrolysis in the presence of Ca^{2+} differ only slightly (19). It would be of interest to compare the kinetic parameters of both enzymes in Factor X activation.

In this paper we present a spectrophotometric assay for Factor IX_a, based on the fact that under suitable conditions the rate of Factor X activation is proportional to the Factor IX_a concentration. The assay can be used to determine Factor IX_a in samples from activation mixtures containing purified Factor IX or in samples from plasma after quantitative activation of Factor IX by incubation with purified Factor XI_a and Ca^{2+}.

A major requirement in an assay for plasma clotting factors is to exclude the effects of inhibitors and activators in plasma. Fig. 5 shows that the amount of Factor IX_a generated is proportional up to a plasma concentration of 40 µl per ml of Factor IX activation mixture. Usage of larger amounts of plasma (defibrinated with thrombin to prevent clotting) results in incomplete activation of Factor IX. This might be due to the influence of protease inhibitors. Possible interference of the spectrophotometric assay by Factors $VIII_a$ and VII_a was excluded by the finding that plasmas congenitally deficient in either of these two factors contain about the same amount of Factor IX as normal reference pool plasma. The slightly lower Factor IX content is also found with the clotting assay, and can be ascribed to individual variations (Table 2). The finding that no Factor IX_a activity arises during activation of Factor IX deficient plasma, further excludes interference in the assay by Factor VII_a or by another unknown Factor X activator.

Factor IX is one of the vitamin K dependent coagulation factors and plasma from patients on oral anticoagulant therapy contains descarboxy proteins, lacking the γ-carboxy glumatic acid residues that are involved in Ca^{2+} dependent binding to the negatively charged phospholipid surface (34). Coumarin plasma with 26% Factor IX in a clotting assay, has Factor IX 31% of normal in the spectrophotometric assay. Apparently, the descarboxy Factor IX molecules present in coumarin plasma do not participate in the reactions of the assay. This is to be anticipated since the activation of Factor X by Factor IX_a takes place at a phospholipid surface and diminished Ca^{2+} dependent binding of descarboxy Factor IX_a will lower the rate of Factor X activation when less lipid bound enzyme is available.

In addition or alternatively, it may be possible that descarboxy Factor IX cannot be activated to descarboxy Factor IX_a by Factor XI_a. In a comparable enzymatic reaction it was found that descarboxy Factor X is very slowly converted to descarboxy Factor X_a by RVV-X (35).

The spectrophotometric assay for Factor IX described here may prove to be useful for determination of Factor IX in patient plasma. The assay incorporates a phospholipid dependent activation reaction, responds to descarboxy Factor IX, and may find application in evaluation of oral anticoagulant therapy.

Addendum

A paper on the clinical application of the Factor IX method reported here is in press. Title and authors: "Use of chromogenic peptide substrates in the determination of clotting Factors II, VII, IX and X in normal plasma and in plasma of patients treated with oral anticoagulants.« M. P. van Dieijen-Visser, J. van Wersch, P. J. Brombacher, J. Rosing, H. C. Hemker and G. van Dieijen. Haemostasis.

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