Stabilization of Thrombin-Activated Porcine Factor VIII:C by Factor IXa and Phospholipid

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The activation of porcine factor X by an enzymatic complex consisting of activated factor IX (factor IXa), thrombin-activated factor VIII:C (factor VIII:Ca), phospholipid vesicles, and calcium was studied in the presence of an irreversible inhibitor of factor Xa, 6-dimethylamino-naphthalene-1-sulfonyl-glutamyl-glycyl-arginyl-chloromethyl ketone (DEGR-CK). The formation of factor Xa was measured continuously by monitoring the increase in solution fluorescence intensity that occurs upon formation of DEGR-factor Xa. Omission of any component from the enzymatic complex reduced the reaction rate to a negligible level. In the presence of fixed excess factor IXa, the velocity of factor X activation was linearly dependent on the concentration of factor VIII:C, and thus, provided a plasma-free assay of factor VIII:C. Activation of factor VIII:C by 0.1 NIH U/ml thrombin in the presence of factor IXa, phospholipid vesicles, and calcium, followed at variable time intervals by the addition of factor X and DEGR-CK, was complete within 5 min, as judged by the fluorometric assay, and resulted in little or no loss of factor VIII:C activity over a period of 20 min; whereas, activation in the absence of either IXa or phospholipid vesicles decreased the half-life of factor VIII:C to approximately 5 min. Analysis of 125I-factor VIII:C-derived activation peptides by sodium dodecyl sulfate polyacrylamide gel radioelectrophoresis revealed identical results, regardless of whether factor IXa and/or phospholipid vesicles were included in the activation, suggesting that the lability of factor VIII:Ca is not due to a major alteration of its primary structure. We conclude that the activated porcine factor VIII:C molecule is stabilized markedly by its interaction with factor IXa and phospholipid.

Factor VIII:C is a protein whose presence is necessary for the normal activation of factor X by the intrinsic pathway of blood coagulation. Several lines of evidence suggest that activated factor VIII:C is a cofactor for the serine protease factor IXa in a reaction that also requires phospholipid and calcium for maximal activity.1–5 Recently, an isolation procedure from this laboratory has been described that results in a 300,000-fold purification of factor VIII:C from porcine plasma.6 This preparation consists of four polypeptide chains, visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with apparent molecular weights of 166, 130, 82, and 76 kDa. N-Terminal sequence analysis suggests that the 130- and 82-kDa peptides are proteolytic products of the 166-kDa polypeptide,7 and affinity chromatography of factor VIII:C on monoclonal anti-factor VIII:C-Sepharose, in the presence and absence of calcium chelating agents, indicates that the 76-kDa polypeptide associates with the 3 largest species noncovalently to form a calcium-dependent complex.8 Factor VIII:C is a procofactor which, in vitro, is converted to the more active cofactor by thrombin. Reaction of porcine factor VIII:C with thrombin causes factor VIII:C activity to increase 60–80 fold in coagulation assays and results in proteolysis of all four peptides. It has been observed, in both purified preparations and plasma, that factor VIII:C is labile after activation by thrombin,9–13 although the cause of this phenomenon is unknown. In this study, we report that the presence of factor IXa and phospholipid significantly stabilizes activated factor VIII:C.

MATERIALS AND METHODS

The following materials were purchased from Sigma Chemical Company, St. Louis, MO: L-α-phosphatidylcholine, type III-E; t-α-phosphatidyl-t-serine (from bovine brain); sulfopropyl-Sephadex C-50 (SP-Sephadex); QAE Sephadex, Q-50; ω-N-benzoyl-t-arginine ethylester-HCl; dextran sulfate; soybean trypsin inhibitor; glycine; bovine serum albumin (RIA grade); e-amino caproic acid; Taipan venom (Oxyuranus scutellatus); semicarbazide; rabbit brain cephalin; factor VII and X deficient bovine plasma; and crude Russell’s viper venom. CH-Sepharose was obtained from Pharmacia Chemicals, Uppsala, Sweden. Lactoperoxidase was purchased from Calbiochem, La Jolla, CA, and was diluted to 20 μg/ml in 0.05 M sodium phosphate, pH 7.4, and frozen in small aliquots at –70°C prior to use. Na125I, 13–17 μCi/μg, was purchased from Amersham Corporation, Arlington Heights, VA. Heparin-Sepharose was a gift from Alpha Therapeutics, Los Angeles, CA. Dextran sulfate-agarose was prepared using cyanogen bromide, according to the method of Kiesler.14 Human factors VIII, IX, and XI deficient plasma were purchased from George King Biomedical, Overland Park, KA. p-Nitrophenyl-guanidinobenzoate (NPGB) was purchased from ICN Pharmaceuticals, Cleveland, OH, and stored at 4°C at a concentration of 2.5 mM in 1 : 4 dimethyl formamide:acetoni-tle (v/v) prior to use. Alcohol dehydrogenase and nicotinamide adenine dinucleotide were obtained from Boehringer-Mannheim, West Germany. 5-Dimethylamino-naphthalene-2-sulfonyl-glutamyl-glycyl-arginyl chloride (DEGR-CK) was a gift from Dr. E. Shaw. Benzamidine hydrochloride was purchased from Aldrich Chemical Company, Milwaukee, WI. Acrlyamide and bis-acrylamide were obtained from Bio-Rad Laboratories, Richmond, CA. N-benzoyl-t-isoleucyl-l-glutamyl-glycyl-arginyl-p-nitroanilide (S-2222) was purchased from Kabi Diagnostica, Stockholm, Sweden. Imidazole and purchased from J. T. Baker, Phillipsburg, NJ and recrystallized before use. Heparin (porcine intestinal mucosa) (1,000 USP U/ml) was purchased from Abbott Laboratories, North Chicago, IL. Celite 545 was purchased from Johns-Manville, Denver, CO. Sodium dodecyl sulfate was

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obtained from Pierce Chemical Company, Rockford, IL. Kaolin was from Merck Company, Rahway, NJ. Polyethylene glycol 8000 was purchased from J. T. Baker, Phillipsburg, NJ.

**Phospholipid**

Phosphatidyl-choline-phosphatidyl-serine (75%/25%) (PCPS) vesicles were prepared by a modification of the method of Barenholz. The concentration of phospholipid in the vesicles was determined by assay for inorganic phosphorus.

**Proteins**

The factor X coagulant protein from Russell's viper venom (RVV-XCP) was isolated as described by Kiesel et al. For some experiments, factor Xa was coupled to CH-Sepharose-4B, according to instructions provided by the manufacturer.

Contact product was prepared as described by Nossel, using human fresh frozen or plasmapheresis plasma, and additionally, was concentrated to approximately 2-5 mg/ml by precipitation from 50% saturated ammonium sulfate solution, resuspended in 0.05 M sodium acetate, pH 5.2, and dialysis against this buffer. Contact product prepared by this method was stable for many months, as judged by assays for activated factor X.

Porcine vitamin K-dependent proteins were isolated from citrated blood of animals heparinized (55 U/kg) prior to venipuncture. All steps were done at 4°C unless otherwise indicated. The blood was drawn into 600-ml evacuated bottles, containing 60 ml 3.8% trisodium citrate, pH 7.5, and 1 mM benzamidine, pH 6.0, which was stored in 0.2 M sodium citrate, pH 7.5, and applied to a 0.15 x 30 cm column of heparin-Sepharose, and a 0.15-0.5 M NaCl gradient in 0.02 M phosphate, 1 mM CaCl2, 50% (v/v) ethylene glycol at 4°C. The resulting activation mix was applied to a 1.5 x 30 cm column of heparin-Sepharose, and a 0.15-1 M NaCl gradient in 0.02 M Tris-Cl, pH 7.4, was applied to SDS-PAGE. The preparation contained 0.85-0.95 active sites/molecule, as judged by titration of samples from several preparations with NPG.

Factor Xa was prepared by passing factor X over Russell's viper venom-factor X coagulant protein bound to Sepharose (RVV-XCP-Sepharose). Factors IX and X and prothrombin were concentrated by dialysis into 80% saturated ammonium sulfate solution and stored as suspensions at 4°C. Factor IXa (0.1 mg/ml) was stored in 0.2 M NaCl, 0.05 M imidazole, pH 6.0, in small aliquots at -70°C. Thrombin (0.1 mg/ml) was stored in small aliquots in 0.4 M NaCl, 0.02 M Tris-Cl, pH 7.5, at -70°C. Factor Xa was stored in 0.15 M NaCl, 0.02 M Tris-Cl, 1 mM benzamidine, pH 7.4, at 4°C and was used within 2-3 days. All other proteins were stable for several months under these conditions, as judged by coagulation assays and SDS-PAGE.

Factor VIII:C was prepared as described and was stable for at least 3 wk when stored in 0.1 M NaCl, 0.01 M histidine, pH 6.0, 5 mM CaCl2, 50% (v/v) ethylene glycol at 4°C. In some cases, it was dialyzed against 0.2 M sodium acetate, 5 mM Ca(NO3)2, pH 6.8, prior to use. Factor VIII:C was radiiodinated using the lactoperoxidase method of Thorell and Johansson. One hundred microliters of 100-200 U/ml Factor VIII:C in 0.2 M sodium acetate, 5 mM Ca(NO3)2, pH 6.8, was reacted with 5 μl of lactoperoxidase, 20 U/ml, 0.002% hydrogen peroxide (v/v), and 200 μCi 125I for 2 min. The reaction was stopped with 0.4 ml 0.1% sodium azide, 6% bovine serum albumin (BSA). The mixture was then applied to monoclonal anti-factor VIII:C (W-3-3) bound to Sepharose and washed with 0.1 M NaCl, 0.01 M histidine, 5 mM CaCl2, 0.15 M NaCl, 0.1% BSA, pH 6.0, to remove unreacted Na125I. Factor VIII:C was eluted with 0.1 M NaCl, 0.01 M histidine, 5 mM CaCl2, 0.1% BSA, 50% ethylene glycol, pH 6.0, and stored in this buffer at 4°C. The labeled factor VIII was also stable for at least 3 wk, as judged by coagulation assay. Assay of 125I-factor VIII:C prior to separation of free 125I revealed an average loss of 25% activity in 3 iodinations. The recovery of activity from the monoclonal antibody column ranged from 60% to 70% of the applied sample, which is similar to the discovery of unlabeled factor VIII:C from this column. The final product remained 30-40-fold activatable by thrombin and contained 1-3 atoms of 125I/factor VIII:C molecule, assuming a clotting activity of 5 U/μg and a molecular weight of 240,000 g/mole.

**Coagulation Factor Assays**

Factors VIII:C and IXa were assayed using human deficient plasmas. Factor X was assayed using bovine factors VII and X deficient plasma, crude Russell's viper venom, and rabbit brain cephalin. Citrated porcine plasma was used as a standard. Thrombin was assayed as described by Owen, using NIH reference lot B-3 as a standard. Factor IXa was assayed using BAEE ester as substrate.

**Ultracentrifuge Studies**

Minimal molecular weights of factor X, IXa, and thrombin were determined in 6 M guanidine hydrochloride in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. Analogs to digital conversion of the signal was done with a Bascom-Turner 8110 electronic recorder, and individual data points were
obtained using a curve tracking digitizer. Analyses were carried out in double sector cells at 20° to 23° using 3-mm solution heights under conditions of meniscus depletion. Partial specific volumes were estimated from amino acid composition, with correction for carbohydrate content. Values were 0.720, 0.717, 0.731 ml/g, respectively, for factors IXa, X, and thrombin. Solution densities were measured by pycnometry. Plots of ln C versus r² were constructed using an Apple III computer and, in all cases, were linear. Weight-average molecular weights for porcine factors IXa, X, and thrombin, obtained by linear least-squares regression, were 45,000, 57,000, and 37,000 g/mole, respectively. Extinction coefficients (E<sub>1%cm</sub>) were measured using the method of Babul and Stellwagen. Values obtained were 15.2, 8.4, and 19.9 for factors IXa, X, and thrombin, respectively. A value of 14.9 was assumed for factor IX.

**Amino Acid Composition and Carbohydrate Analysis**

Amino acid composition was determined from 24-hr 6N HCl hydrolysates using high performance liquid chromatography of the amino acids and detection of the orthophthalaldehyde derivatives.

**Fluorometric Assays**

The activation of factor X was studied by measuring the increase in solution fluorescence intensity at 23° when DEGR-CK irreversibly inhibits factor Xa. A Perkin-Elmer MP43 fluorometer was used with a 430-nm cutoff filter. Wavelengths were set at the maxima of the excitation and emission spectra of DEGR-Xa and were 335 nm and 520 nm, respectively. To a cuvette containing 1.6 ml, 0.15 M NaCl, 0.02 M Tris-Cl, and 2 mM CaCl₂, pH 7.5, were added solutions containing PCPS vesicles, factor IXa, factor VIII:C, and thrombin, as indicated in figure legends. Stability studies were done by reacting factor VIII:C with thrombin for variable times in the presence of calcium, plus or minus factor IXa and PCPS vesicles. Factor VIII:C activity was then measured by adding factor IXa and/or PCPS vesicles, if originally missing, followed 0.5 min later by DEGR-CK and factor X. The velocity of factor X activation was measured graphically from the continuous trace of increasing fluorescence intensity at the region where the rate of change of intensity with respect to time was linear (see Fig. 1). The factor VIII:C concentration was then calculated from a standard curve of velocity, plotted as a function of nominal VIII:C concentration, constructed by activation for 30 sec in the presence of calcium, PCPS vesicles, and factor IXa, followed by addition of DEGR-CK and factor X. In experiments where the addition of <sup>125</sup>I-factor VIII:C was used, 0.25-ml aliquots were removed prior to the addition of DEGR-CK and factor X. These samples were then added to 25 μl of 10% SDS, preheated to 90°, and heated for 3 min at 90°C for SDS-polyacrylamide gel radioelectrophoresis (SDS-PAGE).

**Chromogenic Assay**

In some experiments, the rate of factor Xa formation measured in the fluorometric assay was compared to a chromogenic assay using S-2222 as a substrate. In the chromogenic assay, a reaction mixture was prepared that was identical to that used for the fluorometric assay, except DEGR-CK was omitted. At various times, an aliquot was added to a solution containing EDTA at a final concentration of 50 mM, which immediately stopped factor Xa formation. A 10-μl aliquot of this solution was then added to 0.25 ml 0.4 mM S-2222 in 0.15 M NaCl, 0.02 M Tris-Cl, 0.1% PEG, 50 mM EDTA, and the rate of para-nitroanilide production was followed spectrophotometrically. Initial rates were determined under conditions in which less than 5% substrate (S-2222) conversion had occurred and were compared to a standard curve using dilutions of a stock solution factor Xa, whose concentration had been determined by active site titration with NPGB.

**Electrophoresis**

Five to fifteen percent gradient slab SDS-PAGE, using the buffer system of Laemmli, was done for both analysis of vitamin K-dependent proteins and autoradiograms of factor VIII:C. For autoradiograms, the gels were dried, without further manipulation, using a slab gel dryer. Autoradiograms were made at ~70°C using Kodak X-Omat AR film and an x-ray intensifying screen. Standard proteins, E. coli β-galactosidase (116,000), BSA (69,000), ovalbumin (43,000), carbonic anhydrase (30,000), and cytochrome C (13,000), were radiolabeled using chloramine T.

**RESULTS**

The reaction of factor Xa with DEGR-CK results in covalent, active-site modification of the enzyme and an increase in solution fluorescence intensity (Fig. 1). By carrying out the activation of factor X in the presence of DEGR-CK, it is possible to continuously monitor the reaction by measuring fluorescence intensity. Figure 1 shows activation of factor X in the presence of calcium, PCPS vesicles, factor IXa, DEGR-CK, and three concentrations of thrombin. Similar results were obtained using <sup>125</sup>I-factor VIII:C. As the thrombin concentration is decreased, a lag phase in the increase in fluorescence intensity becomes more pronounced, presumably due to a decrease in the rate of activation of factor VIII:C. In the absence of thrombin, the lag phase was further prolonged, but eventually, significant factor X activation was observed. The nature of this phenomenon is currently under investigation. In the absence of either factor IXa, factor VIII:C, PCPS vesicles, or calcium, the reaction proceeded to a negligible extent (data not shown).

By using thrombin concentrations in which factor
VIII:C activation is fast, relative to the activation of factor X by the entire enzymatic complex, it is possible to study the factor X activation as a function of nominal factor VIII:C concentration. In these experiments, thrombin concentrations from 0.1 to 1 NIH U/ml were chosen. Varying amounts of factor VIII:C were thrombin-activated for 30 sec in the presence of fixed factor IXa, calcium, and phospholipid; DEGR-CK and factor X were added, and the velocity was measured graphically as a function of nominal factor VIII:C concentration. Figure 2 shows that, under the conditions used, the velocity of factor X activation is linearly dependent on the factor VIII:C concentration to approximately 0.05 U/ml and then shows saturation. This saturation phenomenon was not entirely due to saturation of the enzymatic complex with factor VIII:Ca, as increasing factor IXa or phospholipid concentrations had relatively little effect on the saturation curve. Instead, the reaction rate of DEGR-CK with Xa becomes limiting, because as the factor VIII:C concentration is increased, the rate of increase in fluorescence intensity becomes identical to that when factor Xa is reacted with DEGR-CK.

The fluorometric assay was used to measure factor VIII:C activity by measuring the velocity of factor X activation under conditions in which it is linearly related to the nominal factor VIII:C concentration. Under these conditions, initial velocity measurements were indistinguishable from rates of factor Xa production measured by S-2222 hydrolysis. This indicates that the fluorometric assay can be used to measure factor VIII:Ca activity. $^{125}$I-factor VIII:C was used in some experiments, so that analyses by SDS-PAGRE could be done. All activity experiments described were also done using unlabeled factor VIII:C, resulting in identical results. When $^{125}$I-factor VIII:C was activated by 0.1 NIH U/ml thrombin in the presence of phospholipid and factor IXa, and then assayed at various times by the addition of DEGR-CK and factor X, it lost approximately 30% of its activity over a period of 40 min (Fig. 3). In contrast, activation of $^{125}$I-factor VIII:C in the absence of either phospholipid or factor IXa, or both, led to a 50% loss of activity in approximately 7 min (Fig. 3). Equivalent results were obtained by using 1 U/ml thrombin.

Preliminary experiments showed that labeling factor VIII:C with $^{125}$I using the fluid-phase lactoperoxidase method resulted in a 25% average loss of clotting activity. Assuming a clotting activity of 5 U/µg for factor VIII:C and an approximate molecular weight of 240 kDa, several iodinations resulted in a range from 1 to 3 iodine atoms/factor VIII:C molecule. $^{125}$I-Factor VIII:C preparations showed radioactivity mainly corresponding to the 82- and 76-kd peptides, with little radioactivity corresponding to the 160- and 130-kd peptides, indicating either that these larger peptides do not incorporate $^{125}$I or that the procedure results in degradation of the 160- and 130-kd peptides to the 82-kd peptide (Fig. 4A). Activation of $^{125}$I-factor VIII:C only in the presence of thrombin resulted in rapid proteolysis of the 82-kd peptide and slower degradation of the 76-kd peptide (Fig. 4E) with concomitant formation of 69-, 44-, and 35-kd peptides. This cleavage pattern is identical to that seen with nonradiolabeled factor VIII:C. When factor VIII:C activation was carried out in the presence of factor IXa (Fig. 4C), phospholipid (Fig. 4D) or both (Fig. 4B), the cleavage patterns, as a function of time of exposure to thrombin, were identical to that seen in the absence of phospholipid and factor IXa.
of factor IXa or phospholipid. This indicates that loss of factor VIII:C activity is not due to major changes in the primary structure of the VIII:C molecule. During these activation experiments, there was less than 10% loss of the mass of factor VIII:C from the system, as judged by counting the radioactivity of the solution. The solutions contained 0.3% ethylene glycol (v/v), resulting from dilution of 125I-factor VIII:C in 50% ethylene glycol into the system. When 125I-factor VIII:C was dialyzed against 0.2 M sodium acetate, pH 6.8, prior to use, there was no loss of factor VIII:C mass when either factor IXa or PCPS vesicles were included in the system. However, in the absence of both of these components, approximately 75% of the radioactivity was lost from solution and the half-life of factor VIII:Ca decreased to less than 1 min (not shown).

**DISCUSSION**

In this article, we report initial investigations concerning the role of factor VIII:C in the factor IXa-catalyzed conversion of factor X to Xa. An isolation procedure for porcine VIII:C, which is free of contaminating proteins, makes it possible to study the molecular events in this activation in greater detail. Current evidence suggests that factor VIII:Ca is a cofactor for factor IXa in the same manner that factor Va is a cofactor for factor Xa in prothrombin activation. According to this hypothesis, factor VIII:Ca binds to the phospholipid surface of the platelet (and/or possibly to a protein receptor) and becomes part of a supramolecular complex with factors IXa, X, and calcium. A problem arising in the approach to the study of the kinetics of factor X activation is the observation that thrombin-activated factor VIII:C loses activity.3, 13 Recently, Hultin and Jesty reported that the loss of factor VIII:C activity is not prevented by inhibitors of thrombin, indicating that loss of activity is not due to further proteolysis by thrombin.13 In addition, they presented a model consistent with extant data in which factor VIII:C simultaneously undergoes thrombin-dependent activation and thrombin-independent inactivation. In experiments shown in Figs. 3 and 4, we used a concentration of thrombin (0.1 U/ml) that apparently leads to activation within 5 min, so that the main effect observable during the course of the study is loss of VIII:C activity. Under these conditions, in the absence of factor IXa and phospholipid, factor VIII:Ca loses most of its activity in 5–10 min (Fig. 3), which is consistent with data from coagulation assays by Hultin and Jesty13 and by Rick and Hoyer.14 In contrast, addition of factor IXa and phospholipid during activation significantly prolongs VIII:C activity (Fig. 3).

Using 125I-factor VIII:C, analysis of the cleavage peptides of the molecule can be examined by SDS-PAGE with increased sensitivity and without the complication of visualizing nonradioactive proteins in the solution. 125I-factor VIII:C has the same thrombin cleavage pattern as unlabeled factor VIII:C6 (Fig. 4E). Protection of factor VIII:C activity by factor IXa or phospholipid does not alter the cleavage pattern of the peptides that are produced during activation (Fig. 4). In addition, the loss of factor VIII:C activity does not appear to be due to adsorption to the reaction vessel wall, as there is no loss of solution radioactivity.

These results do not agree with the recent work of Fulcher et al.,4 who have proposed that spontaneous loss of human factor VIII:C activity is due to proteoly-
sis of a 92-kd polypeptide. The cause of this discrepancy is not known.

Given these data, it seems reasonable to propose that, upon activation of factor VIII:C by thrombin in the presence of factor IXa, phospholipid, and calcium, peptides are generated that are bound in the enzymatic complex and serve as a cofactor for factor IXa in solution. Presently, neither the peptides that constitute porcine VIII:Ca nor the process that causes the lability of factor VIII:Ca are known. A possibility for the latter includes conformational changes that are not allowed when factor VIII:Ca is bound to phospholipid and factor IXa.

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