Cleavage and Activation of Human Factor IX by Serine Proteases

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Human factor IX circulates as a single-chain glycoprotein. Upon activation in vitro, it is cleaved into disulfide-linked light and heavy chains and an activation peptide. After reduction of activated $^{125}$I-factor IX, the heavy and light chains are readily identified by gel electrophoresis. A direct, immunoradiometric assay for factor IXa was developed to assess activation of factor IX for proteases that cleaved it. The assay utilized radiolabeled antithrombin III with heparin to identify the active site and antibodies to distinguish factor IX. After cleavage of factor IX by factor Xla, factor VIIIa–tissue thromboplastin complex, or the factor X-activating enzyme from Russell’s viper venom, antithrombin III bound readily to factor IXa. Cleavage of $^{125}$I-factor IX by trypsin, chymotrypsin, and granulocyte elastase in the presence of calcium yielded major polypeptide fragments of the sizes of the factor Xla-generated light and heavy chains. Kallikrein did not cleave the zymogen. Nonactivation cleavage was noted by thrombin, but only in the absence of calcium. When the immunoradiometric assay was used to assay trypsin-cleaved factor IX, the product bound antithrombin III, but not maximally. After digesting with insolubilized trypsin, clotting activity confirmed activation. In contrast, incubation of factor IX with elastase (Takaki et al, J Clin Invest 71:1706, 1983) or chymotrypsin did not lead to generation of an antithrombin III-binding site, despite their digestion of $^{125}$I-factor IX into heavy and light chain-sized fragments. In evaluating activation of factor IX, physical evidence of activation cleavages does not necessarily correlate with generation of an active site.

MATERIALS AND METHODS

Materials

Chemicals were purchased from the following sources: acrylamide, Coomassie Blue R-250, sodium dodecyl sulfate, N, N, N', N'-tetramethylethylenediamine, and lactoperoxidase-glucose oxidase reagent (Enzymobead) from Bio-Rad, Richmond, Calif; bovine...
albumin (fraction V), dithiothreitol, heparin, rabbit brain cephalin, bovine pancreatic trypsin attached to either beaded agarose or polyacrylamide beads, and human α1-antitrypsin (α1-protase inhibitor) from Sigma, St Louis; N, N'-methylene-bis-acrylamide and chloramine T from Eastman Kodak, Rochester, NY; bovine pancreatic trypsin treated with N1-tosylamide-2-phenylethyl chloromethyl ketone from Worthington Biochemical Division, Millpore Corporation, Freehold, NJ; bovine pancreatic chymotrypsin A4 from Boehringer Mannheim, Indianapolis; sodium [125I] iodide from New England Nuclear, Boston; and kaolin from Fisher Scientific, Pittsburgh, Pa. All other chemicals were the best grade available from commercial sources.

Human factor IX was prepared as described previously,† as were human granulocyte elastase,² human thrombin,² and mouse monoclonal antifactor IX antibody.⁵ The factor X-activating enzyme from Russell’s viper venom,⁶ activated human factor VII,⁸ dilisopropyl fluorophosphate-treated human tissue thromboplastin,¹¹ human antithrombin III (1 mg/mL) complexed to heparin,¹² and immunopurified goat antibody to human factor IX were generously provided by Dr Walter Kisiel (University of Washington).⁷ Activated bovine¹³ and human¹⁴ factors Xa were gifts from Dr Kotoku Kurachi (University of Washington), and kallikrein derived from human plasma prekallikrein was from Dr Kazuo Fujikawa (University of Washington).⁵ Activated bovine⁵ and humanFactor VIIa were gifts from Dr Richard Freeman (University of Washington), and kallikrein derived from human plasma prekallikrein⁵ was from Dr Kazuo Fujikawa (University of Washington). All protein solutions were stored at −80 °C. Throughout, Tris-buffered saline was 0.15 mol/L NaCl in 50 mmol/L Tris, pH 7.75.

**Radioiodinations**

Labeling of factor IX and of antithrombin III–heparin by the chloramine T method was carried out as previously described; labeling of goat antibody to factor IX was done identically, except the reaction was carried out for 60 seconds. Labeling with the lactoperoxidase glucose-oxidase reagent was performed according to the manufacturer’s instructions; to the beads swelled in distilled water at 4 °C was added 50 μL 0.2 mol/L sodium phosphate, pH 7.2, 25 μL of protein solution, 10 μL sodium [125I] iodide (1 mCi), and 25 μL 1% d-glucose. With factor IX and antithrombin III–heparin, the reactions were performed for ten minutes at ambient temperature, but for 25 minutes with goat antibody to factor IX. Reaction mixtures were desalted on 10-μL columns of Sephadex G-25 “medium,” which had been prewashed with 2 mL 10% bovine albumin in Tris-buffered saline and equilibrated and eluted with Tris-buffered saline.

**Enzyme Digests**

Unless otherwise specified, reactions were performed at 37 °C in a final volume of 40 μL containing 215 ng factor IX diluted in Tris-buffered saline, with bovine albumin and CaCl₂ added to final concentrations of 0.1% and 2.5 mmol/L, respectively. Reactions were carried out in 1.5-μL polypropylene microfuge tubes that had been preincubated with 1% bovine albumin in Tris-buffered saline for 15 minutes at ambient temperature to minimize protein adsorption. When [125I]factor IX was included, it was present in trace amounts at a level of 2,000 to 10,000 cpm per reaction mixture. Enzyme-substrate ratios used are detailed in the Results section. Reactions were terminated either by addition of denaturant prior to gel electrophoresis or by addition of α1-antitrypsin and EDTA, as described below for the radioimmunometric assay. For each enzyme used, optimal conditions were sought in order to provide reproducible levels of cleavage and/or activation. Nevertheless, enzyme activities did vary somewhat with age (depending, for example, on storage conditions and number of times thawed and refrozen); therefore, under the defined conditions, results were not always maximal. However, data presented are representative of several incubations.

**Polyacrylamide Gel Electrophoresis**

Electrophoresis was carried out in the presence of sodium dodecyl sulfate on 10% polyacrylamide gels (6 mm × 10 cm) prepared and run as described by Weber and Osborn* or on 5% or 7.5% gels prepared by modifying the quantities of acrylamide and N, N'-methylene-bis-acrylamide in the method described. Preparation of protein solutions for electrophoresis was done by the method of Fairbanks et al,¹⁷ except that glycerol was used in place of sucrose and bromophenol blue instead of pyronin Y as the tracking dye. Electrophoresis was routinely carried out until the leading edge of the dye had migrated 8 cm. Incubations were performed overnight at 37 °C. For the preparation of nonreduced samples, dithiothreitol was eliminated from the incubation mixture. Unstained gels of radiolabeled proteins and fragments were routinely sliced with an electric gel slicer (Bio-Rad, Model 195) and 2-mm segments counted in a Nuclear Chicago Gamma Counter (Model 1085, 80% efficiency).

**Factor IXa-Antithrombin Binding Assay**

Purified goat antifactor IX, which contained 19 mg/mL protein and 800 Bethesda inhibitor U/mL, was diluted 1:500 in 0.1 mol/L NaHCO₃, pH 9.5, and 100 μL incubated for at least one hour at 37 °C in microtiter wells (Imulon-I Removawell strips, Dynatech, Alexandria, Va). Wells were then washed three times with Tris-buffered saline containing 0.1% albumin. After incubating unlabeled factor IX with enzymes as described above (see **Enzyme Digests**), reactions were terminated by adding 4 μg α1-antitrypsin and EDTA to a final concentration of 5 mmol/L and incubating for ten minutes at 37 °C. Then, [125I]-antithrombin III was added and the mixture incubated at 37 °C for an additional ten minutes; in general, the amount of labeled inhibitor added was calculated such that, on a molar basis, there was a slight excess of active inhibitor over the factor IXα. The factor IXα-antithrombin III mixture was finally diluted to 125 μL in Tris-buffered saline with bovine albumin added for a final concentration of 0.1%, and 100 μL of this dilution was transferred to the antibody-coated wells and incubated at ambient temperature for two hours. During this incubation, the wells were individually precounted. After incubation, the wells were washed three times with 0.1% albumin in Tris-buffered saline and counted again. Percentage binding was calculated from the ratio of final count to precount. Typically, pipetting and counting error in duplicate determinations averaged less than 1.5% of the counts added.

**Factor IXα Clotting Assay**

Factor IXα clotting activity was determined by a “nonactivated” factor IX assay with deficient substrate plasma, but without kaolin, as modified from a previously described assay procedure.⁶ The incubation mixtures contained 100-μL aliquots each of diluted enzyme digest (100 ng factors IX + IXα), Tris-buffered saline, rabbit brain cephalin (dissolved according to manufacturer’s instructions, aliquoted, and stored at −20 °C, then warmed to 37 °C and diluted fivefold with 0.15 mol/L NaCl before use) and factor IX-deficient plasma in a siliconized glass tube (10 × 75 mm). After a one-minute equilibration at 37 °C, 100 μL of 35 mmol/L CaCl₂ was added, and the clotting time determined by titping.

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*References to “antithrombin III” in this article all refer to the inhibitor with heparin as eluted."
**RESULTS**

**Radiolabeling of Antithrombin III**

The initial challenge in development of the factor IXa assay was incorporating sufficient specific radioactivity, yet minimizing inactivation of the inhibitor by the labeling procedure. In preliminary experiments, it was found that antithrombin III was progressively inactivated by conditions used in radiolabeling, thus limiting the useful incorporation of iodine 125. Labeled preparations were screened by reacting 125I-antithrombin III with an excess of either thrombin or factor IXa and the nonreduced products electrophoresed on 5% polyacrylamide gels in sodium dodecyl sulfate. The radioactivity in the thrombin–antithrombin III or factor IXa–antithrombin III complexes was then compared with that in the unbound antithrombin III. Labeling using the chloramine T method, with only a ten-second exposure to a low concentration of oxidant, yielded, as a typical example, a 125I-antithrombin III preparation which, besides having a low specific radioactivity, could only bind 43% to thrombin. To minimize oxidative damage, the solid-phase, lactoperoxidase-glucose oxidase system was used. A ten-minute incubation, for example, yielded a preparation of which 66% could bind to factor IXa (Fig 1); the range over several labels was 45% to 70%.

Of particular importance was the fact that the specific activity was typically 40-fold greater than that obtained during the ten-second chloramine T reaction. For experiments in this study, this enzymatic labeling procedure was used exclusively.

**The Immunoradiometric Factor IXa Assay and Incubation With Factor XIa**

The specificity for factor IX was provided by an insolubilized antibody; the activated form was identified by being covalently bonded to the labeled inhibitor. For the factor IXa assay, the polystyrene wells were coated with polyclonal goat anti-factor IX antibody, or in some cases, antifactor IX mouse monoclonal antibody. Optimal conditions for coating of the wells were assessed by the binding of 125I-factor IX to wells that had been coated with various dilutions of the antibody. With the goat antibody, maximal binding of 125I-factor IX occurred in an antibody dilution range from 1:100 to 1:1,000. Coating wells at 37 °C for one hour yielded a greater binding capacity than at 4 °C for 72 hours, especially at 1:100 antibody dilution. For all assays, goat antibody was routinely diluted 1:500 and incubated in the wells for one hour at 37 °C before washing and use. There was no significant difference between the binding of 125I-factor IX and that of factor XIa-activated 125I-factor IX.

The degree of activation was determined by preincubation of the factor IX reaction mixture with 125I-antithrombin III, followed by its binding to the antibody-coated well. As a control, with nonactivated factor IX, the radioactivity bound to the well was less than 1% of that observed after maximal activation by factor XIa. As a further control, purified thrombin was incubated with 125I-antithrombin III and tested in the assay system. Binding was about 5% maximal, but the low level appeared to be due to trace contamination of the thrombin with factor IXa, as factor IX antigen was present at a level of 0.01 U/mL as measured by an immunoassay using the monoclonal antibody to factor IX. The presence or absence of α1-antitrypsin had no effect on binding of 125I-antithrombin III to factor IXa, but this inhibitor was included to prevent digestion of the antithrombin by the less specific proteases. Likewise, calcium concentrations of 2.5, 5, and 10 mmol/L in factor XIa-activation incubations gave essentially the same amount of factor IXa by 125I-antithrombin III binding. To limit the final concentration of EDTA, which was added to terminate the activations, 2.5 mmol/L calcium was selected for most experiments.

In order for a factor IXa assay to be readily usable and quantitative, it should show a linear response over a range of factor IXa concentrations. A representative

**Fig 1.** Sodium dodecyl sulfate-5% polyacrylamide gel electrophoresis patterns of labeled antithrombin III and factors IX or IXa. 
(A) An eight-fold excess of factor Xla-activated factor IX was incubated for ten minutes at 37 °C with 125I-antithrombin III. After electrophoresis, the gel was sliced into 2-mm sections and the radioactivity measured (solid circles). (B) Radiolabeled antithrombin III alone (solid circles) and 125I-factor IX (open circles) were run on separate gels as controls. Migrations were from left to right toward the anode.
dilution curve of factor IXa generated by activation with factor XIa (Fig 2) shows linearity over a wide range of factor IXa, with a constant amount of $^{125}$I-antithrombin III. When another preparation of $^{125}$I-antithrombin III with higher specific radioactivity was used, a tenfold greater sensitivity was achieved (not shown), although 55% of the inhibitor would not bind to excess enzyme. For the purposes of this study, however, the greater sensitivity was not necessary. For the standard curves, ratios of factor IXa to labeled antithrombin III varied from 1.2- to 1.6-fold excess of factor IXa over a wide range of concentrations (not shown in Fig 2). However, assumptions of (1) the factor IXa concentration as based on the initial concentration of the proenzyme and the degree of cleavage in parallel experiments, (2) uniform reactivity of the labeled inhibitor that binds to factor IXa, and (3) equilibrium for the antigen–antibody interaction all limit the accuracy of calculated stoichiometry.

In early experiments using the assay, it was demonstrated that activation of factor IX by bovine factor XIa$^1$ gave the same time-dependent increments of $^{125}$I-antithrombin III binding as human factor XIa$^5$; cleavage patterns on gels were likewise identical. Bovine factor XIa was used routinely to produce the “maximum activatable” human factor IX in the current study.

Throughout this study, the assay described was used to measure the amount of $^{125}$I-antithrombin III–factor IXa complex bound to goat antifactor IX-coated wells in the presence of excess inhibitor. After thorough rinsing to remove the excess, the measured radioactivity remaining in the well was counted. The calculated percentage of counts added was therefore dependent on the degree to which the $^{125}$I-antithrombin III was added in excess and the specific activity of the $^{125}$I-antithrombin III preparation. Consequently, for comparisons of different proteases, binding values were normalized to factor IX maximally activated by factor XIa under the conditions described and run simultaneously. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reduced samples of factor IXa, the maximal digestion by factor XIa, under the conditions utilized, converted at least 70% of intact factor IX to heavy and light chains (Fig 3A). The amounts of radioactivity within the different peaks are summarized in Table 1.

**Incubation With Russell’s Viper Venom Protease**

Cleavage of factor IX by the factor X-activating enzyme from Russell’s viper venom revealed a pattern of reduced samples on gels that differed from that of factor XIa-cleaved factor IX. As shown in Fig 3B, the intermediate peak migrated further toward the anode, indicating a smaller size. The intermediate, resulting
from cleavage by the venom activator, was smaller by two to three slices on each of eight separate cleavages when compared with the less anodal migration of the intermediate as observed in all of over 30 factor XIa or six factor VIIa activation cleavages studied by electrophoresis of reduced samples. As shown in Table I, however, the amount of 125I-antithrombin III complexed to the product of activation of factor IX by the protease from Russell's viper venom was comparable to that measured after activation by factor XIa. Lower enzyme-substrate ratios produced the same peaks as shown in Fig 3B, but with less cleavage of intact factor IX at 60 minutes.

Incubation With Factor VIIa-Tissue Thromboplastin

Cleavage of factor IX by factor VIIa-tissue thromboplastin at molar enzyme (factor VIIa) to substrate ratios ranging from 1:1 to 1:20 (see Fig 3C) yielded patterns qualitatively the same as those observed upon digestion with factor XIa (Fig 3A). However, when comparing the gel patterns of factor Xla-activated and factor VIIa-activated 125I-factor IX, there were some quantitative differences in the radioactivity present in corresponding peaks (Table I). At enzyme-substrate ratios of 1:20 and below, less than 50% cleavage of native factor IX was observed on sodium dodecyl sulfate-polyacrylamide gels. Under the conditions defined in Table I, binding of 125I-antithrombin III averaged 20% less than when factor IX was maximally activated by factor XIa (see Table I). In control experiments in which tissue thromboplastin and factor VIIa were added to factor Xla-digested factor IX prior to its incubation with antithrombin III, a 10% decrease in binding of the inhibitor was observed. A similar effect was seen with the tissue thromboplastin alone. Thus, components of the extrinsic activator complex interfered somewhat with maximal 125I-antithrombin III binding.

Incubation With Trypsin

Cleavage of factor IX by soluble bovine trypsin yielded a gel pattern with the most prominent peaks corresponding to the radioactivity present in the native factor IX and heavy chain-sized fragments. When the incubation was carried out in the absence of added calcium. It is noteworthy that upon continuing the digestion for up to 120 minutes (in the presence of calcium), factor IX was resistant to further cleavages by trypsin (Fig 4A). A similar digestion pattern was seen when the incubation was carried out in the absence of added calcium. In either case, about one tenth of the radioactivity in the heavy chain-sized fragment was consistently lower, and in the light chain-sized fragment, consistently higher than seen following digestion by factor XIa (Table I).
digestion by trypsin led to the generation of an active activation, degradation or both. Analysis by the immunoradiometric assay, the binding of antithrombin III increased by 39% (Table 1) to 81% of maximum upon addition of factor Xla to a trypsin-incubated factor IX. Conversely, when factor Xla-activated factor IX was incubated with trypsin for 60 minutes, the binding of 125I-antithrombin III decreased by 22%, suggesting that some degradation occurred.

To determine whether generation of factor IXa procoagulant activity paralleled generation of the ability to bind antithrombin III during digestion of factor IX by trypsin, the study was extended to include the use of trypsin insolubilized on polyacrylamide beads. In this way, the enzyme could be removed and the digestion stopped by centrifugation. The supernatant so produced was tested in the factor IXa clotting assay. Trypsin insolubilized on agarose was less satisfactory in this experiment, because the agarose was found to adsorb greater amounts of 125I-factor IXa. A time-course digestion of factor IX with polyacrylamide-bound trypsin was analyzed by correlating (1) the appearance of heavy and light chain-sized fragments upon polyacrylamide gel electrophoresis of reduced samples in sodium dodecyl sulfate, (2) the generation of ability to bind 125I-antithrombin III, and (3) the development of procoagulant activity in a factor IXa coagulation assay; results are presented in Table 2. Antithrombin III binding paralleled the appearance, and subsequent slow disappearance, of the heavy chain-sized fragment. Furthermore, the increase of coagulant activity paralleled generation of antithrombin III binding sites.

The immunoradiometric assay was then used to determine whether trypsin-activated factor IX could be further activated by incubation with factor Xla and, conversely, what effect digestion by trypsin would have on factor Xla-activated factor IX. Addition of factor Xla to a 60-minute trypsic digest of 125I-factor IX and incubation for an additional 60 minutes, markedly altered the gel pattern from that which resulted simply from digestion by trypsin for the full 120 minutes (Fig 4A). The major difference when trypsin was followed by a second 60-minute incubation with factor Xla was a twofold greater quantity of heavy chain-sized fragment and less intermediate. In the immunoradiometric assay, the binding of antithrombin III increased from 39% (Table 1) to 81% of maximum upon addition of factor Xla to a trypsin-incubated factor IX. Conversely, when factor Xla-activated factor IX was incubated with trypsin for 60 minutes, the binding of 125I-antithrombin III decreased by 22%, suggesting that some degradation occurred.

Table 2. Activation of Factor IX by Polyacrylamide-Bound Trypsin

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>125I-Antithrombin III Bound (%)</th>
<th>Clotting Time (sec)</th>
<th>cpm in Heavy Chain Peak (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8</td>
<td>&gt; 200</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10.2</td>
<td>55.6</td>
<td>14.3</td>
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<td>15</td>
<td>13.1</td>
<td>50.7</td>
<td>16.6</td>
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<tr>
<td>30</td>
<td>16.9</td>
<td>47.0</td>
<td>18.1</td>
</tr>
<tr>
<td>60</td>
<td>15.3</td>
<td>39.5</td>
<td>18.3</td>
</tr>
</tbody>
</table>

Aliquots were removed at the indicated times and assayed. The binding and clotting assays were performed on the same reaction mixture. Digestion of factor IX containing trace 125I-factor IX was done in parallel and gels were run to determine percent heavy chain, as in Table 1.

*As opposed to Table 1, percent antithrombin III bound is reported as the raw percent; a simultaneous control of factor IX maximally activated by factor Xla gave 26% binding of the 125I-antithrombin III added.

† The clotting assay consisted of 100 µL each of sample aliquot, Veronal buffer, factor IX-deficient plasma, and rabbit brain cephalin suspension, incubated at 37 °C for one minute; then, 100 µL 0.035 mol/L CaCl2 was added and the clotting time determined (see Materials and Methods).
To determine if the coagulant activity after incubation with trypsin was influenced by traces of the pancreatic enzyme that might have remained in the supernatant of the incubation mixture, assays were carried out on dilutions to which an excess of $\alpha_{1-}$antitrypsin had been added. Although the inhibitor did not decrease the procoagulant activity of factor XIa-activated factor IX, its addition resulted in a seven-second longer clotting time (compared with that in Table 2) on factor IX cleaved by trypsin insolubilized to polyacrylamide beads. Furthermore, dilution curves with the inhibitor, which were somewhat steeper in trypsin-activated factor IX, became parallel with factor XIa-activated factor IX. In separate experiments, factor IXa activities, in the presence of excess $\alpha_{1-}$antitrypsin, were diluted from factor IXa obtained by both factor XIa and trypsin-cleaved factor IX. The starting reagents were adjusted in concentration to give comparable levels (6% to 7% binding) of $^{125}$I-antithrombin III in the immunoradiometric assay. Dilution curves of these incubation mixtures showed the same amount of coagulant activity in the factor IXa clotting assay.

**Incubation With Chymotrypsin**

Incubation of factor IX with chymotrypsin in the presence of calcium was followed by reduction and gel electrophoresis in the presence of sodium dodecyl sulfate and yielded a pattern similar to that observed with the other enzymes (Table 1). The predominant peaks corresponded in size to the intact protein, one or two intermediates, and heavy and light chains (Fig 4B). In addition, lower molecular weight peptides were observed. The relative amount of radioactivity in the light chain-sized fragment was about the same as with the factor XIa-generated peptide but less than that resulting from digestion with trypsin (Table 1). As was the case when trypsin was used, the heavy chain-sized fragment resulting from chymotryptic cleavage was present at much lower levels than after activation by factor XIa (Table 1); most of the remaining radioactivity appeared instead in the higher molecular weight intermediates. Like digestion with trypsin, incubation of factor IX with chymotrypsin in EDTA at variable concentrations of calcium (50 $\mu$mol/L to 3.2 mmol/L) showed moderately greater levels of low-molecular weight fragments when the calcium concentration was below 1 mmol/L. With the chymotrypsin digestion at the higher calcium levels, however, there was additional radioactivity in the intact factor IX peak (as opposed to the intermediate peak seen with trypsin, above).

The cleavage pattern of reduced samples on polyacrylamide gels in the presence of sodium dodecyl sulfate suggested that chymotrypsin can activate factor IX. However, the immunoradiometric factor IXa assay showed that the cleavage product did not bind antithrombin III at all (Table 1).

A further question of interest is whether cleavage by chymotrypsin precluded subsequent activation by factor XIa. Comparisons of the gel patterns in Fig 4B shows that initial digestion by chymotrypsin did not prevent subsequent cleavage of intermediates by factor XIa, leading to an increase in radioactivity in the heavy chain-sized peak. Nevertheless, as with trypsin, the percentage of radioactivity in the region of the light chain was at least 50% greater than when digestion was performed by factor XIa alone (Table 1). These gel patterns suggest, therefore, that there was degradation of calcium-stabilized factor IX by chymotrypsin to fragments of the size of the light chain leading to partial inactivation of factor IX. This, in fact, was found to be the case. After a second incubation and factor XIa cleavage for 60 minutes, chymotrypsin-digested factor IX was able to bind $^{125}$I-antithrombin III; however, the binding was only 60% of that obtained after maximal activation of intact factor IX by factor XIa alone. When the last experiment was carried out in reverse, ie, factor XIa activation followed at 60 minutes by additional chymotrypsin for an additional 60 minutes, the binding of $^{125}$I-antithrombin III was reduced by 55%.

**Incubation With Granulocyte Elastase**

As previously studied, the gel patterns of elastase-digested factor IX showed primarily heavy and light chain-sized fragments.\(^5\) Under conditions with more limited digestion (Fig 4C), intact factor IX and an intermediate of the same size as that formed during digestion with factor XIa were also observed. As with chymotrypsin, elastase (even after limited digestion) did not activate factor IX, as determined by its inability to bind $^{125}$I-antithrombin III (Table 1). Because it was noted that there was greater elastase degradation of factor IX in the absence of calcium than with other proteases, these studies were extended by varying the calcium concentration to determine if a critical level of calcium were necessary for more specific cleavages. In the absence of calcium (ie, in 5 mmol/L EDTA), only 11.5% of the total radioactivity migrated at the location of the heavy chain-sized fragment, whereas 25% of the radioactivity existed in low-molecular weight fragments running beyond the bromphenol blue marker dye (on 7.5% polyacrylamide gels). As the calcium concentration was increased from 0.05 mmol/L to 0.8 mmol/L (in the absence of EDTA), the amount of
Incubation With Thrombin and Kallikrein

After incubation of factor IX with thrombin for 60 minutes at a molar enzyme-substrate ratio of 1:10 in the presence of calcium, the gel electrophoresis pattern showed no indication of cleavage of the factor IX molecule (Table I). In the absence of calcium, however, an additional prominent peak migrating just beyond the intact molecule (larger than the intermediate obtained during activation by factor XIa) was observed. The fragment represented 25% of the radioactivity present on the gel; a small peak containing 10% of the counts migrated between the factor IXa light chain and marker dye positions. After a four-hour incubation without calcium, 24% of counts remained in the intact factor IX peak, 50% in the large intermediate position, and 26% was in the low-molecular weight peptide peak migrating at 56 mm (slice 28) on the 10% polyacrylamide gel. The same thrombin fragments were observed when the reducing agent was omitted. There was no evidence of heavy and light chain-sized fragments after incubation with thrombin, and thus, no evidence of activation. This was confirmed by the lack of binding of antithrombin III (Table 1).

Incubation of factor IX with kallikrein for 60 minutes, with or without calcium, at a molar enzyme-substrate ratio of 1:5 gave no indication of cleavage of the factor IX molecule after reduction (Table I). On polyacrylamide gels in the presence of sodium dodecyl sulfate, all of the radioactivity ran at the position of the intact molecule. In addition, no I25I-antithrombin III bound to factor IX after incubation with kallikrein (Table 1).

DISCUSSION

The direct factor IXa assay utilizes the interaction between antithrombin III and the active enzyme form. In the presence of heparin, inhibition occurs in seconds and a covalent bond is formed; the enzyme–inhibitor complex does not dissociate on gel electrophoresis in detergent18 (see Fig 1). Labeling of the antithrombin III was provided by iodination, and gentle conditions were required to minimize inactivation of the inhibitor.5 In order to distinguish I25I-antithrombin III-factor IXa complexes from other enzymes that would bind to the labeled inhibitor, monospecific antibodies were used. These include polyclonal goat5 and monoclonal mouse8 antifactor IX preparations adsorbed to microtiter wells at high pH. Relatively short incubations were required, and the entire assay could be set up and run in four hours.

The specificity of the assay was validated by the lack of binding of inhibitor when only native (unactivated) factor IX or control samples without factor IX were present. Nonspecific binding of the inhibitor to the wells was likewise negligible. Although the sensitivity of the assay was more than sufficient for the purposes of this study, it is possible that it could be markedly enhanced by using a labeled antibody to antithrombin III, for example, similar to assays for plasmin and elastase.20 With the physiologic concentration of factor IX being around 5 μg/mL,6 such a modification would probably be needed to detect trace activation at this level.

When factor IXa was diluted over a wide range of concentrations, a linear dose response was obtained in the immunoradiometric assay. A potential limitation of the factor IXa assay relates to the requirement of a double logarithmic plot to achieve linearity (see Fig 2). This may be due to partial damage of the antithrombin III during labeling. Alternatively, it may reflect steric characteristics of the interactions of the complex with the antibody bound to the surface of the well, a lack of equilibrium conditions, or both. Thus, it is possible that a standard curve based on dilution of factor XIa-activated factor IX would not necessarily quantitatively reflect the amount of factor IXa present under certain conditions, such as partial activation (ie, the presence of excess intact or inactivated zymogen).

Considering other measurements of factor IXa coagulant activity, it is noted that nonactivated clotting assays using factor IX-deficient plasma are dependent on the net effects of several enzymatic reactions. Furthermore, following digestion of factor IX with a protease of broad specificity, determination of the factor IXa activity by a clotting assay requires first the functional, if not physical, removal of the protease to avoid effects on other factors. Coupled intrinsic factor X activation assays have also been used to determine factor IXa activity, either with chromogenic substrates specific for factor Xa2 or by generation of trichloroacetic acid-soluble, 3H-labeled factor X activation peptide.21 Although factor X is the physiologic sub-

1This may be analogous to inactivation of an homologous inhibitor, α1-antitrypsin, upon iodination, although a specific Met oxidation could also account for the latter.19
strate for factor IXa, there is, in these assays, still a dependence on modified factor VIII. The latter's reactive is highly variable, as up to 50-fold enhancement can occur with the traces of thrombin; in addition, factor VIII is more labile after modification. There are no specific low-molecular weight inhibitors or chromogenic substrates for factor IX, although thioester peptides show some selectivity.

In contrast to functional assays, physical measurements have also been studied. These include generation of 125I-labeled heavy and light chains or the 3H-labeled activation peptide of factor IX; the latter is capable of providing kinetic parameters in isolated systems. Although these are direct measurements, neither of them necessarily reflects generation of procoagulant activity. As shown in studies with granulocyte elastase, cleavage can occur with inactivation. Thus, in order to study the effects of other proteases on factor IX, the direct assay of factor IXa was required.

Among previously described activators of factor IX in vitro, factor Xla and the factor X-coagulant protein from Russell's viper venom yielded comparable amounts of antithrombin III binding in the immunoradiometric assay (see Table I). This would be predicted from the similar levels of heavy chain noted on parallel samples, in which 125I-factor IX was included, after reduction and electrophoresis (Table 1). With Russell's viper venom-activated 125I-factor IX, the intermediate peak, migrating between the intact factor IX and the heavy chain of factor IXa, was of lower molecular weight than that observed following activation of factor IX by factor Xla or factor VIIa. This is due to the lower rate of the venom enzyme's cleavage of the bond between the light chain and the activation peptide. The clinical relevance of an initial Arg-Ala cleavage is underscored by the recent identification of a mutation leading to substitution at this Arg in the factor IXa from a patient with one type of hemophilia B.

As shown by Osterud and Rapaport, the factor VIIa–tissue thromboplastin complex yielded qualitatively similar patterns on gels to those seen with intrinsic activation. In addition, antithrombin III binding was comparable (Table 1), especially considering the mild reduction of binding by factor Xla-activated factor IX noted in the presence of tissue thromboplastin. Thus, after intrinsic or extrinsic activation, an inactive species, factor IXa, is first formed as a result of the cleavage of Arg-Ala bond between the light chain and the activation peptide. One quantitative difference routinely seen was that this species accumulated to a greater extent during activation by the extrinsic system complex compared with activation by factor Xla (Table 1). For some factor VIIa-factor IX ratios studied, up to one third of the counts were in the intermediate peak, as previously observed. This may reflect slower cleavage of the Arg-Val bond during extrinsic (as opposed to intrinsic) system activation. Nevertheless, the final active enzyme, factor IXa, would be the same. The intermediate peak has also been noted in reduced samples of extrinsically activated 125I-human factor IX. In contrast, extrinsic activation of bovine factor IX occurs without formation of an intermediate species, presumably due to more rapid cleavage of the Arg-Val bond. This apparently represents an important species difference in extrinsic activation.

A novel interaction investigated in the current study is that of factor IX and trypsin. Activation of factor IX by trypsin had not previously been shown, but the pancreatic enzyme has been reported to activate several other clotting factors. For example, factor XI is partially activated by trypsin, although at least one additional cleavage occurs. By using insolubilized trypsin, it was possible to remove the trypsin and, along with α1-antitrypsin, to eliminate its effects on functional assays. Antithrombin III binding was then demonstrated. As shown with degraded forms of thrombin, antithrombin III binding (or, for that matter, esterase activity) does not always reflect procoagulant activity. Using the factor IXa clotting assay, however, coagulant activity was found in trypsin-digested factor IX. Furthermore, this activity paralleled both heavy chain formation and antithrombin III binding (see Table 2 and Fig 4A) and was as expected from an equivalent amount of factor Xla-activated factor IX.

It is apparent from the amino acid sequence of human factor IX that there are numerous potential cleavage sites for trypsin throughout the protein chain. These include the bonds cleaved during activation by factor Xla. Had other cleavages occurred at either the carboxy-terminal end or within the activation peptide, however, they would not have been distinguished. Although trypsin partially activated factor IX (Table 2), there was a relative lack of rapid additional cleavages leading to either prevention of activation or subsequent inactivation. It should also be noted that tryptic hydrolysis can be useful in distinguishing abnormal factor IX molecules. Prolonged tryptic digestion in the absence of calcium yielded a series of peptides, including one in which a mutant amino acid (His instead of Arg at position 145) prevented cleavage in a hemophilic factor IX molecule.

Human factor IX was not cleaved by bovine thrombin in calcium, but the cleavage described in the current study using human proteins only occurred without calcium. Similar fragments were observed with thrombin-digested bovine factor IX by Marlar.
and Seegers, likewise without calcium. Although Seligsohn et al noted cleavage of \(^{125}\)I-factor IX by kallikrein in the presence of partially purified factor VII and calcium, and Osterud et al had shown a slow activation of factor IX by kallikrein, the preparations in the current study showed no cleavage by kallikrein in a more isolated system, even at a high enzyme-factor IX ratio.

It can be concluded that, in calcium-stabilized human factor IX, regions around the bonds cleaved by factor Xa during activation are exposed and readily susceptible to noncoagulation serine proteases. Furthermore, it is apparent that the remainder of the factor IX molecule is relatively resistant to major degradative cleavages. Because of a lack of specificity, trypsin can only partially activate factor IX. On the other hand, digestion by chymotrypsin or elastase yields heavy and light chain-sized fragments, but does not produce activation; it would be predicted that they would not generate a free amino terminus at Val\(_{191}\) (see Discussion, ref. 5). The retention of a degree of intact structure is confirmed by partial generation of antithrombin III binding sites after factor XIa was added to the digestion mixtures. Thus, physical evidence of cleavage is an inadequate assessment of activation and, as noted above, indirect determinations also have their limitations. However, activation of factor IX in isolated systems can be measured by use of a direct, immunoradiometric assay based on binding of factor IXa to the antithrombin III–heparin complex. Inasmuch as this direct approach assesses binding of a relatively nonspecific, active site probe, it could theoretically overestimate activation compared with the more stringent structural requirements for specific, macromolecular, enzyme-substrate binding of factor IXa to factor X.

The factor IXa immunoradiometric assay does provide a simple procedure to detect intact active site components, and it is capable of demonstrating that trypsin, a serine protease with broad specificity, can activate factor IX. The assay is well suited to examine activation-like cleavages by cellular neutral proteases to screen for as yet undescribed systems that may activate or degrade factor IX during coagulation. In addition, a modification of this approach has been used to detect factor IXa in commercial, therapeutic factor IX concentrates, as possibly relevant to their thrombogenic potential. Both the assay and the patterns of protease digestion will also be useful in distinguishing specific structural defects among the various abnormal factor IX molecules in patients with hemophilia B.

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Cleavage and activation of human factor IX by serine proteases

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