Inhibition of Human Factor IX\textsubscript{a} by Human Antithrombin*

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A procedure is presented for the purification of Factor IX from human plasma. The final product is homogeneous as judged by disc gel electrophoresis and sodium dodecyl sulfate gel electrophoresis. Furthermore, it is completely free of other coagulation component activities.

Factor IX is converted to its enzymatically active form by the addition of small quantities of Factor IX\textsubscript{a} in the presence of calcium ions. This activated species is added to purified antithrombin-heparin cofactor and the interaction is studied in the presence and absence of heparin. Antithrombin-heparin cofactor is found to be a progressive, time-dependent inhibitor of Factor IX\textsubscript{a} and neutralizes approximately 57% of this enzyme's proteolytic activity within 30 min. The addition of heparin dramatically accelerates the rate of this interaction with virtually complete inhibition of Factor IX\textsubscript{a} occurring within 15 s. Sodium dodecyl sulfate gel electrophoresis of reduced and nonreduced proteins indicates that antithrombin-heparin cofactor functions as a potent inhibitor of Factor IX\textsubscript{a} by forming an undissociable complex with the enzyme which is stable in the presence of denaturing or reducing agents (or both). This complex represents a 1:1 stoichiometric combination of enzyme and inhibitor. Heparin increases the rate of formation of this complex without affecting its dissociability or stoichiometry.

The coagulation cascade is composed of a series of linked proteolytic reactions which ultimately lead to the generation of thrombin. At each stage of this mechanism, a parent zymogen is converted to a corresponding serine protease which catalyzes a subsequent zymogen-serine protease transition. Once evolved, thrombin releases two pairs of fibrinopeptides from fibrinogen and permits the resulting fibrin to polymerize into an insoluble clot. Resolution of this meshwork is, in part, accomplished via an analogous cascade of reactions which culminates in the production of plasmin. This serine protease is capable of hydrolyzing the fibrin clot.

A variety of plasma components oppose the action of serine proteases of the coagulation-fibrinolytic mechanism, but each protein has a narrowly restricted pattern of inhibitor specificity (1 to 3). It was initially believed that antithrombin heparin cofactor shared this property and could only neutralize thrombin and Factor X\textsubscript{a} (4).

In a previous communication, this laboratory has reported the purification and mechanism of action of human antithrombin-heparin cofactor (5). We have shown that this component neutralizes the activity of human thrombin by formation of an enzyme-inhibitor complex via an active center serine-reactive site arginine interaction and that heparin dramatically accelerates the rate of this reaction. On the basis of the biochemical mechanism of antithrombin-heparin cofactor's action and our knowledge that this component could inactivate thrombin as well as Factor X\textsubscript{a}, we predicted that most, if not all, serine proteases of the coagulation-fibrinolytic mechanism would be neutralized by this inhibitor and that heparin would accelerate each of these interactions (6). Thus, we proposed that this plasma component and its naturally occurring mucopolysaccharide cofactor could represent a critical control point in modulating the activities of the hemostatic mechanism (5, 6).

To test this concept, we have previously examined the interactions of human antithrombin-heparin cofactor with human Factor XI\textsubscript{a} as well as human plasmin and have shown that these serine proteases are inactivated in this, heretofore unsuspected, fashion (6, 7). In this communication, we demonstrate that human antithrombin-heparin cofactor progressively inhibits human Factor IX\textsubscript{a} in the absence of heparin and that the rate of inactivation is virtually instantaneous when the acidic mucopolysaccharide is present.

MATERIALS AND METHODS

Chemicals

All chemicals employed were reagent grade or better.

Plasma

Human plasma was obtained by plasmaphoresis of normal donors, utilizing (0.44 g of citric acid/1.32 g of trisodium citrate/1.47 g of dextrose/100 ml of H\textsubscript{2}O) as anticoagulant. Protein purifications were initiated within 2 hours of plasma collection.

Proteins

Bovine serum albumin, phosphorylase, chymotrypsinogen, ovalbumin, and cytochrome c were purchased from Sigma Corp. Human fibrinogen was obtained from AB Kabi, Stockholm, Sweden. Protamine sulfate was provided by Eli Lilly Co.

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Heparin
Heparin employed to activate antithrombin was obtained from Organon. Heparin utilized to prepare heparin-Sepharose was obtained in crude form (Stage I) from the Wilson Chemical Corp. It was purified as described by Lindahl et al. (8).

Column Chromatographic Materials
DEAE-cellulose, dry hydroxyapatite, and polyacrylamide P-150 were purchased from Bio-Rad. Sepharose 4B and Sephadex G-25 were obtained from Pharmacia Fine Chemicals. Heparin-Sepharose was prepared as previously described (5), except that this matrix was washed extensively with 0.6 M sodium chloride in 0.05 M Tris-HCl (pH 7.5) and then equilibrated with 0.15 M sodium chloride in 0.05 M Tris-HCl (pH 7.5) prior to use.

Measurements of Protein Concentrations
Protein concentrations were determined by absorbance measurements at 280 nm by the method of Lowry et al. (9), using bovine serum albumin as a standard (Protein Standard Solution, Armour Pharmaceutical Co.).

Measurements of Coagulation Component Activities
Factor IX activity was assayed according to the method of Bachmann et al. (12). All computations were performed according to methods as described by Bennett and Franklin (22). In most instances, mean values are given with their associated standard errors.

RESULTS AND DISCUSSION
Purification of Human Factor XI
Human Factor XI, was partially purified from plasma by DEAE-cellulose chromatography, cellulose adsorption-elution, hydroxyapatite chromatography, and polyacrylamide P-150 gel filtration, according to a minor modification of the method of Amir et al. (16), as utilized in our laboratory (6).

Purification of Human Antithrombin-Heparin Cofactor
Human antithrombin-heparin cofactor was isolated from plasma by the method of Rosenberg et al. (6).

Purification of Human Factor IX
Step I. DEAE-cellulose Adsorption-Elution, Barium Citrate Adsorption-Elution, Differential Ammonium Sulfate Precipitation, and Filtration through Sephadex G-25—One and one-half liters of human plasma were fractionated by DEAE-cellulose batch adsorption-elution, barium citrate adsorption-elution, and differential ammonium sulfate precipitation according to the procedure of Shapin et al. (17) for the isolation of prothrombin. Three minor modifications of this technique were utilized. Firstly, the DEAE-cellulose effluent was assayed for Factor IX activity only and the most active fractions were pooled. Secondly, the ammonium sulfate precipitations were performed at saturations of 33 and 66%. Thirdly, the final precipitate of approximately 200 absorbance units of protein was dissolved in 0.2 M potassium phosphate (pH 6.8) and desalted by filtration at 4° through a Sephadex G-25 column (1.4 x 40 cm), previously equilibrated with this buffer.

Step II. Hydroxyapatite Chromatography—Two to three milliliters of the Step I product, containing approximately 60 absorbance units of protein, were applied to a column packed with heparin-Sepharose (0.8 x 5.0 cm) previously equilibrated with 0.15 M sodium chloride in 0.05 M Tris-HCl (pH 7.5). The chromatographic matrix was washed with 0.2 M sodium chloride in 20 ml of 0.05 M Tris-HCl (pH 7.5) and the protein was eluted with 0.50 M sodium chloride in 0.05 M Tris-HCl (pH 7.5).

Step III. Heparin-Sepharose Chromatography—Eight to ten ml of the Step II product, containing approximately 7 absorbance units of protein, were applied to a column packed with heparin-Sepharose (0.8 x 5.0 cm) previously equilibrated with 0.15 M sodium chloride in 0.05 M Tris-HCl (pH 7.5). The flow rates were maintained at 2 to 3 ml/hour by gravity flow. Column fractions were assayed for Factor IX activity. Those containing the highest Factor IX specific activity, without detectable Factor X, were pooled and stored at –70°.

Gel Electrophoresis
The disc gel electrophoretic procedure of Davis (18) as modified by Rosenberg and Waugh (19) was used. Samples were applied with 10% glycerol added. The sodium dodecyl sulfate gel electrophoretic system of Fairbanks et al. (20) was employed. The gels were stained for protein with Coomassie brilliant blue and molecular weights were calculated according to the technique of Weber and Osborn (21) utilizing cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin, and phosphorylase as marker proteins. Each molecular weight represents an average of at least two separate determinations.

Diagnosis
All dialysis bags (Union Carbide) were boiled in 3% sodium carbonate, rinsed with distilled water, reboiled in distilled water, and rerinsed prior to use.

Statistical Computations
All computations were performed according to methods as described by Bennett and Franklin (22). In most instances, mean values are given with their associated standard errors.
antithrombin-heparin cofactor, and fibrinogen. In addition, disc gel electrophoresis of these preparations revealed a single band with 86% of the applied Factor IX activity located precisely at this position (Fig. 1). Furthermore, a single molecular species was usually observed (five preparations) when Step III products were examined by sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents (Fig. 1).

On occasion (two preparations), a second component of slightly greater mobility was observed on sodium dodecyl sulfate gel electrophoresis in the presence of reducing agent. This molecular species comprised 10 to 15% of the applied protein and appeared to be a partially proteolized form of Factor IX. However, these preparations were not utilized in subsequent studies of the interaction of Factor IXa with antithrombin-heparin cofactor.

Our technique represents the first method for obtaining this human coagulation protein in pure and stable form. A partial purification of human Factor IX has previously been reported (23). It is a relatively simple, rapid and efficient procedure. Steps I and II of this isolation method have been utilized previously by our laboratory in the purification of human Factor X (24). Both of these steps were originally described by other investigators as isolation techniques useful in preparing partially purified human prothrombin and partially purified human Factor X (17, 25). We have made only minor modifications in their methodology. Step III of this isolation technique is similar to that used in the preparation of bovine Factor IX (26). However, the original procedure was designed to separate bovine Factor IX from bovine prothrombin since bovine Factor X was removed at an earlier stage in the fractionation process. In our preparative method, heparin-Sepharose chromatography (Step III) is utilized to separate human Factor IX from human Factor X, since human prothrombin is eliminated from our final products by hydroxylapatite chromatography (Step II). Thus, it is not surprising that the heparin-Sepharose chromatographic procedure used for the purification of bovine Factor IX required substantial modification to accomplish our objective. This alteration included a change in buffer composition, as well as a revision of the ionic strength elution schedule.

The conversion of enzymatically inactive zymogen to Factor IXa was accomplished by the addition of Factor XIa and calcium ions. Approximately 1.0 to 6.0 µg of partially purified Factor XIa were required to fully activate 4.5 to 180.0 µg of Factor IX. The conversion was conducted at 37° in 0.01 M Tris-HCl buffer (pH 7.5) with an added ionic strength that varied from 0.15 to 0.40 NaCl (CaCl2 concentration was fixed at 0.025 M with remainder contributed by sodium chloride). The generation of active enzyme was complete at approximately 30 min.

This activation process was examined by sodium dodecyl sulfate gel electrophoresis in the absence of reducing agents (not shown). During a 60-min period of incubation with Factor XIa, Factor IX of molecular weight 58,700 ± 240 almost completely disappears in conjunction with the emergence of Factor IXa, of apparent molecular weight 40,000 ± 100 (Fig. 3A, Gel 1).1

This second minor component is believed to be a proteolyzed form of Factor IX since the addition of Factor IXa converts it to a new molecular species with the same molecular weight as Factor IXa, as judged by unreduced sodium dodecyl sulfate gel electrophoresis.

The staining intensity of Factor IXa is considerably less than that of its parent zymogen when equivalent amounts of both proteins are examined by sodium dodecyl sulfate gel electrophoresis.

This conversion of zymogen to serine protease was also monitored by sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents (not shown). In this electrophoretic system, Factor IX was observed to migrate as a single component of apparent molecular weight 61,700 ± 220. Prior to the emergence of significant amounts of Factor IXa, activity, the zymogen was partially converted to two new electrophoretic species of apparent molecular weights 45,000 ± 150 and 17,000 ± 100, respectively. The heavier molecular weight component vanished and was replaced by an electrophoretic species of apparent molecular weight 27,000 ± 400 as well as a lightly staining component of apparent molecular weight ~9000 (Fig. 3B, Gel 2).

Therefore, activation of Factor IX appears to involve an initial scission in the single polypeptide chain of this zymogen to yield an intermediate with two smaller polypeptide chains bridged by S-S bond(s). The heavier polypeptide chain of the intermediate is subsequently cleaved during formation of the active enzyme with the release of a polypeptide fragment. Of course, generation of smaller polypeptides might not be observed by sodium dodecyl sulfate gel electrophoresis but would not grossly alter the overall nature of this proteolytic reaction.

This conversion process is in reasonable accord with the detailed studies of Fujikawa et al. (27) which employed the bovine form of this zymogen. Indeed, the apparent molecular weights of the heavy and light chains of human Factor IXa are in excellent agreement with values obtained by these investigators for the individual chains of the bovine enzyme. However, the apparent molecular weights of human Factor IX and the human intermediate are approximately 15 to 30% higher than those obtained by equilibrium centrifugation of their bovine counterparts (27). Furthermore, there are similar systematic discrepancies between the sum of the apparent molecular weights of the final human activation products and their precursors, Factor IXa, and intermediate.

Proteins can exhibit anomalous mobilities on sodium dodecyl...
sulfate gel electrophoresis due to a high content of carbohydrate which significantly reduces the amount of bound detergent. This results in a reduction in electrophoretic mobility and a higher apparent molecular weight (24). Bovine Factor IX and bovine intermediate are known to have a large amount of carbohydrate attached to their polypeptide backbone (97). Thus, their homologous human counterparts can be expected to behave anomalously on sodium dodecyl sulfate gel electrophoresis (Table I). The true molecular weights of each of these components will need to be established by a more generally valid technique such as equilibrium centrifugation.

Interaction of Factor IXα with Antithrombin-Heparin Cofactor—The interaction of Factor IXα with antithrombin-heparin cofactor was examined in the presence and absence of heparin. The final concentrations of Factor IXα, antithrombin-heparin cofactor, and heparin (when utilized) were 33 μg/ml, 48 μg/ml, and 1.83 units/ml, respectively. All studies were conducted at pH 7.5, ionic strength 0.150, and 24°C.

To establish the relative stability of our enzyme preparations, Factor IXα was incubated for 30 min under standard conditions. As shown in Curve A of Fig. 2, no significant loss in the proteolytic activity of Factor IXα was detected.

To demonstrate that Factor IXα as inactivated by antithrombin-heparin cofactor, the enzyme and inhibitor were mixed together and the resulting solution was assayed sequentially. As shown in Curve B in Fig. 2, a time-dependent decay in Factor IXα activity occurred with only 43.4 ± 1.1% of its initial value remaining after 30 min of incubation with the inhibitor.

To determine whether heparin accelerated the Factor IXα-antithrombin interaction, the inhibitor was incubated with its mucopolysaccharide cofactor prior to the addition of the enzyme and the resulting solution was immediately assayed. However, the presence of heparin required a modification of our usual procedure for quantitating Factor IXα. The measurement of this enzyme’s proteolytic activity was completely dependent upon the extent to which Factor IXα can trigger terminal zymogen-serine protease transitions of the coagulation cascade. Heparin is known to instantaneously inhibit these events via its interaction with antithrombin-heparin cofactor. Therefore, a valid determination of Factor IXα activity requires that this mucopolysaccharide be neutralized prior to assay. This was accomplished by the addition of protamine sulfate to incubation mixtures before the Factor IXα activity of these solutions was quantitated.

Several control experiments demonstrate the validity of this approach. First, we have shown that heparin has no direct effect on Factor IXα activity when our modified assay protocol is followed. For example, Factor IXα was initially incubated with heparin for 15 to 60 s and protamine sulfate was subsequently added. The final concentrations of Factor IXα, heparin, and protamine sulfate were 33 μg/ml, 1.83 units/ml, and 4.0 units/ml, respectively. The solvent conditions utilized were those described above. The enzymatic activity of this mixture was compared to a control in which the Factor IXα was incubated at an identical concentration for an equal period of time in the same solvent. No significant difference in Factor IXα activity was noted (97.8 ± 0.7 versus 100 ± 2.3%).

To analyze the interaction of Factor IXα with heparin and antithrombin-heparin cofactor, the inhibitor was preincubated with the mucopolysaccharide for 30 s at 24°C before the addition of enzyme. After an additional 15-s period of incubation, protamine sulfate was admixed at a final concentration of 66 μg/ml and 8.0 units/ml, respectively. Antithrombin-heparin cofactor was incubated with heparin for 30 s at final concentrations of 96 μg/ml and 3.66 units/ml, respectively. Equal volumes of both solutions were subsequently mixed and immediately assayed for Factor IXα activity. These values were compared to a control in which the enzyme was incubated at an identical final concentration for an equal period of time in the same solvent. Only a small difference in Factor IXα activity was observed (91.2 ± 0.4 versus 100 ± 2.3%).
Fig. 3. Sodium dodecyl sulfate gel electrophoretic analysis of human Factor IXa-human antithrombin interactions in the presence and absence of heparin. A, at the time indicated, aliquots were removed, denatured in the absence of reducing agents, and examined by the sodium dodecyl sulfate gel electrophoretic method of Fairbanks et al. (19). The samples which were applied to the gel contained 16 μg of protein. The gel matrix was established at 1 (per cent bisacrylamide) + (per cent acrylamide) = 7% and C (100 (per cent bisacrylamide/H)) = 2.5%. Anodal migration was downward. Gel 1 represents Factor IXa(IXa); Gel 2 shows antithrombin-heparin cofactor (AT); Gels 3 and 4 depict the interaction of enzyme and inhibitor after 10 and 45 min of incubation, respectively; Gel 5 reveals the extent of this interaction after 15 s of incubation in the presence of heparin (H). See text for additional experimental details. B, the electrophoretic analysis was conducted exactly as described above except that the samples were denatured in the presence of the reducing agents, 20 μm of dithiothreitol and 1% β-mercaptoethanol. In addition, samples with 16 μg of protein were applied to each gel. Gel 1 represents antithrombin-heparin (AT) cofactor; Gel 2 shows Factor IXa(IXa); Gel 3 depicts the interaction of enzyme and inhibitor after 15 s of incubation in the presence of heparin (H). See text for additional experimental details.

The inhibitor and enzyme were mixed at final concentrations of 33 and 48 μg/ml, respectively. This concentration ratio was chosen so that a significant excess of inhibitor (30% molar excess) would be present at completion of this reaction in order to avoid secondary proteolysis of the complex (5). The solvent conditions utilized were pH 7.5, ionic strength 0.155, and 24°C. Aliquots were sequentially removed, immediately denatured, and treated as described in Fig. 3 prior to their analysis by sodium dodecyl sulfate gel electrophoresis in the absence of reducing agents.

In this electrophoretic system, antithrombin-heparin cofactor and Factor IXa migrated as single components with apparent molecular weights of 65,500 ± 200 and 40,000 ± 100, respectively. When these proteins were incubated together, the enzyme band gradually disappeared while the inhibitor band significantly decreased in intensity. Simultaneously, a new species emerged, with an apparent molecular weight of 89,700 ± 300 (Fig. 3).

The interaction of antithrombin-heparin cofactor and Factor IXa was also studied in the presence of heparin (2.16 units/ml). The protein concentrations, conditions of incubation, and analytic methods were identical to those described above. Gel 5 of Fig. 3A reveals that formation of the new molecular species was complete within 15 s. The apparent molecular weight of this component was identical to that obtained in the absence of heparin.

There are compelling reasons for believing that this new species is the Factor IXa-antithrombin complex. First, it emerged in conjunction with the disappearance of electrophoretic bands representing enzyme and inhibitor. Second, its rate of appearance was proportional to the degree of enzyme inactivation. However, the apparent molecular weights of the initial reactants (complexes ~90,000 versus reactants ~105,000). This type of discrepancy was not observed when the plasmin-antithrombin complex was studied with similar techniques (complex ~142,000 versus reactants ~144,000).

On occasion, proteins exhibit anomalous mobilities on sodium dodecyl sulfate gel electrophoresis. This phenomena is due either to large amounts of carbohydrate covalently attached to the polypeptide backbone or to preservation of a special macromolecular conformation in denaturing solvents (28, 29). Thus, the low apparent molecular weight of the interaction product may be caused by a unique orientation of reactants within the complex or by the interiorization or carbohydrate present on either enzyme or inhibitor. It is of interest that with other serine proteases of the intrinsic coagulation cascade. However, the true molecular weights of these complexes will have to be ascertained by other techniques such as analytic ultracentrifugation or light scattering.

The Factor IXa-antithrombin interaction was also examined by sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents. The protein concentrations, conditions of incubation, and analytic methods were otherwise identical to those previously utilized. In this electrophoretic system, the inhibitor migrated as a single component of apparent molecular weight 63,700 ± 200. Factor IXa was observed to consist of a heavy chain of apparent molecular weight 27,200 ± 360 and a light chain of apparent weight 17,000 ± 100 (Fig. 3B, Gel 2). When these proteins were mixed together, the heavy chain of Factor IXa disappeared in conjunction with a significant reduction in the staining intensity of antithrombin-heparin cofactor. In addition, a new species was noted with an apparent molecular weight of 81,200 ± 300. However, no change was observed in the staining intensity of the light chain of Factor IXa. If heparin was present (2.16 units/ml), the transformation of components was instantaneous (Fig. 3B, Gel 3B, but if heparin was absent, this alteration of electrophoretic pattern was only gradually apparent (not shown).
These results further substantiate the interaction of Factor IX, with antithrombin-heparin cofactor and the acceleration of this process by heparin. In addition, the data also demonstrates the formation of the undissociable complex via interactions between the inhibitor and the heavy chain of the enzyme. This is to be expected, since the heavy chain of bovine Factor IX bears the active serine center (27) and this protein of other proteolytic enzymes has been shown to be of predominant importance in forming complexes with antithrombin-heparin cofactor (5, 7). Furthermore, the apparent molecular weight of the reduced enzyme-inhibitor complex is in reasonable agreement with the sum of the apparent molecular weights of the individual constituents (complex ~93,000 versus reactants ~90,000). Thus, the molar stoichiometry of the Factor IX,-antithrombin interaction appears to be 1:1. The anomalous apparent molecular weight obtained for the nonreduced Factor IX,-antithrombin complex must be due to some unique property of the light chain of the enzyme expressed during the interaction process.

Thus, Factor IX, like Factor X, Factor XI, thrombin, and plasmin is slowly but progressively inactivated by antithrombin-heparin cofactor in the absence of heparin and virtually instantaneously neutralized by the inhibitor in the presence of this anticoagulant. On the basis of this cumulative evidence and the known mechanism of action of antithrombin-heparin cofactor, one could reasonably expect that the remaining serine protease of the intrinsic coagulation cascade, Factor XII, would be inactivated by this inhibitor and that heparin would accelerate the speed of its neutralization. We have recently completed a study of the Factor XII,-antithrombin interaction and have confirmed this prediction. This data will be reported in a subsequent communication.

The interaction of heparin and antithrombin-heparin cofactor with virtually all of the enzymes of the coagulation-fibrinolytic system implies that this inhibitory mechanism may be of paramount physiological importance in modulating the activity of the hemostatic process. Indeed, heparan sulfate, a mucopolysaccharide which possesses some anticoagulant activity and is chemically related to heparin, recently has been found on the surface of platelets and endothelial cells (30, 31). The presence of this substance, coupled with the unique properties of this inhibitor, suggests that antithrombin-heparin cofactor might be selectively activated, in vivo, at the blood-surface interface where it could rapidly neutralize serine proteases of the coagulation-fibrinolytic mechanism. The interaction would provide a critical barrier against the action of the hemostatic system and would inextricably link surface properties of arteries, veins, capillaries, and platelets with the maintenance of blood fluidity.
Inhibition of human factor IXa by human antithrombin.
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