The Isolation and Characterization of a Specific Antibody Population Directed against the Thrombin-Antithrombin Complex

Herbert K. Lau and Robert D. Rosenberg

From The Sidney Farber Cancer Institute, Beth Israel Hospital, and the Harvard Medical School, Boston, Massachusetts 02115

We have raised antisera against human thrombin-antithrombin complex in rabbits as well as in goats and have chromatographed the respective γ-immunoglobulin fractions on thrombin-antithrombin-Sepharose as well as on antithrombin-Sepharose. The specific antibody population obtained was utilized to construct a double antibody radioimmunoassay capable of measuring as little as 30 ng/ml of this component. The immunoreactivities of the major related plasma components, prothrombin and antithrombin, were, respectively, ~13,000-fold and ~100,000-fold less than that of the thrombin-antithrombin complex on a molar basis. Reactivities of prothrombin activation intermediates as well as thrombin which are present in only trace amounts were also minimal. With the exception of Factor Va-antithrombin, other hemoestatic enzyme-inhibitor complexes exhibited either none or barely detectable levels of immunoreactivity. The Factor Va-antithrombin interaction product was ~320 times less reactive than the thrombin-antithrombin complex on a molar basis.

The radioimmunoassay was employed to assess the importance of antithrombin as a plasma thrombin inhibitor under conditions approaching the in vivo situation. The results demonstrated that this component is responsible for the neutralization of virtually all of the added thrombin. However, the rate of this interaction within plasma is ~2½-fold less than that predicted from purified systems.

The generation of thrombin represents a pivotal event in the coagulation of blood. Once formed, this serine protease may act upon its multiple protein substrates such as Factor V, Factor VIII, Factor XIII, and fibrinogen, or upon critical cellular elements such as platelets or endothelial cells (1-5). Alternately, the enzyme can be neutralized by naturally occurring protease inhibitors. The latter process has been examined in considerable detail by a variety of laboratories (6-8). Most investigators have concluded that the plasma protein, antithrombin, is the major antagonist of thrombin action (6, 9, 10). However, other groups have suggested that various additional plasma proteins may play a primary role in the neutralization (11, 12). All of these studies have employed purified components, or utilized relatively high concentrations of reagents, or both. This is to be contrasted with the biologically relevant situation in which thrombin is generated at very low concentrations, i.e. $10^{-10}$ M to $10^{-11}$ M, and where a variety of endogenous substances may dramatically influence the rate of interaction of this enzyme with specific protease inhibitors.

To examine the in vivo control mechanisms which govern thrombin neutralization, it is necessary that methods be developed for monitoring this process as it occurs within the blood. Given the low level of enzyme inhibited under normal conditions, we have devoted our efforts to the immunochemical detection of thrombin-antithrombin complex. Earlier studies by other investigators have shown that antisera against this enzyme-inhibitor interaction product can be obtained but that these antibody populations cross-react extensively with prothrombin or antithrombin (13). In this communication, we describe a radioimmunoassay for the measurement of nanogram quantities of thrombin-antithrombin complex which is only minimally influenced by normal in vivo concentrations of prothrombin or antithrombin. In addition, we provide a detailed analysis of the specificity of this technique vis-à-vis other enzyme-antithrombin interaction products. Furthermore, we employ this method to demonstrate that the addition of thrombin to plasma at physiologically relevant levels results in the preferential neutralization of the enzyme by antithrombin, albeit at a slower rate than would be predicted from purified systems.

**EXPERIMENTAL PROCEDURES**

Column Chromatography Materials. DEAE-cellulose and dry hydroxyapatite were purchased from Bio-Rad. Sephadex G-25, Sephadex G-100, Sephadex G-200, Sepharose 4B, and QAE-Sephadex were obtained from Pharmacia Fine Chemicals. Prothrombin, thrombin, antithrombin, and thrombin-antithrombin complex were isolated and characterized by the method of Porath, et al. (15). The attachment of prothrombin, thrombin, or antithrombin was conducted at $4^\circ$C in 0.2 M sodium bicarbonate, pH 8.5. Approximately 0.5 mg of either prothrombin, thrombin, or antithrombin was added per ml of "packed" Sepharose 4B. The thrombin-antithrombin complex was covalently linked to the above resin in a similar manner except that environmental conditions were established at $4^\circ$C and 0.5 M NaCl in 0.2 M sodium bicarbonate pH 7.2. In addition, only 0.2 mg of this interaction product was added per ml of "packed" Sepharose, 4B. Furthermore, this chromatographic matrix was washed with 0.5 M NaCl in 0.01 M acetic acid, pH 2.4 prior to use. Under the above conditions, 2% of $12^\text{C}$ labeled prothrombin, thrombin, antithrombin or thrombin-antithrombin complex were bound to cyanogen bromide activated resin.

Chemicals. Blood plasma was purchased from Aldrich Chemical Company. The derivatization reagent, $N_{-}$-$N'$-dicyclohexylcarbodiimide/2-propanol/1-hydroxybenzotriazole/methylimidazole was kindly provided by Dr. David King. Center for Blood Research, Boston, Mass., and utilized to prevent enzymatic degradation of the thrombin-antithrombin complex (15). All other chemical agents employed were reagent grade or better.

Leech Protein and Snake Venom. Russell's Viper Venom was utilized to activate Factor X. This material was prepared by the method of Kapper, et al. (16) and was kindly provided by Dr. Bruce Opper, Boston, Mass. Edible eel venom was purchased from Sigma and purified by the technique of Fraata, et al. (17).

*This work was supported by the National Institutes of Health, Grants HL3-19131-06, PO1 CA 19688-03, and by a Grant from the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address, the Department of Biochemistry, the University of Hong Kong.

§ An Established Investigator of the American Heart Association.

To whom reprint requests should be addressed.

1 "Experimental Procedures" are presented in the miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 960 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M1748, cite authors, and include a check or money order for $1.00 per set of photocopies.
Specific Antisera for Thrombin-Antithrombin Complex

Crude brain isozim was obtained from Pasteur (Basel, Switzerland) and fractionated by affinity chromatography on thrombin-Sepharose 4B. The purified material was obtained by using protein A-Sepharose 4B followed by ion exchange chromatography on DEAE-cellulose.
revealed a single band with a molecular weight of 88,000 corresponding to the thrombin-antithrombin complex (Inset A). Appropriate assays indicated that no detectable levels of either enzyme or inhibitor were present within this material. The recovery of thrombin-antithrombin complex averaged 45% of the theoretically predicted amount formed within the initial incubation mixture. Peak 2 eluted at an added ionic strength of 0.07. SDS-gel electrophoretic analysis of this material revealed a single band at a molecular weight of 62,000 which corresponded to excess free antithrombin (not shown). Esterolytic assays confirmed this assignment.

Purified thrombin-antithrombin complex was employed to immunize four rabbits and two goats. The absence of trace amounts of thrombin and antithrombin within this preparation permitted us to raise antibody populations that were specific for the interaction product rather than the initial reactants (see below). Bleedings obtained from the various animals were processed individually to produce the corresponding IgG fractions. Each of these preparations was initially chromatographed on a column of thrombin-antithrombin attached to Sepharose 4B in order to isolate antibody population specifically directed against this interaction product. Antibody populations adsorbed to this matrix were harvested as described under “Experimental Procedures.” These fractions were filtered through columns of antithrombin-Sepharose 4B. The latter chromatographic step eliminated antibody population directed against thrombin-antithrombin complex but capable of cross-reacting with antithrombin. This approach increased the specificity of our antibody preparations for the thrombin-antithrombin complex and allowed us to select IgG populations with the highest avidity for this component.

When the final preparations were analyzed, it was apparent that all animals immunized with thrombin-antithrombin complex produced antibodies specifically directed against this material. Two rabbits and one goat (R27, R30, and G184) consistently provided antibody populations with the highest specificity and greatest avidity for thrombin-antithrombin complex. Samples from two separate bleedings are examined in detail throughout this communication. The remaining three animals generated fractions that exhibited a 1/2- to 5-fold reduction in specificity for thrombin-antithrombin complex or d-thrombin or thrombin and antithrombin.

The affinity chromatographic process was analyzed by determining the characteristics of antibody populations derived from R27 or R30 antisera but isolated at various steps during the above fractionation procedure. A double antibody radioimmunoassay was employed to determine the specificity of antibody within the various fractions that is directed against the thrombin-antithrombin complex. The degree of specificity was established by comparing the molar concentrations of prothrombin, antithrombin, and thrombin-antithrombin complex needed to displace 50% of the immunoprecipitable 125I-thrombin-antithrombin counts from an antibody preparation. The data demonstrated that: (a) IgG fractions obtained from R27 or R30 antisera were 80- to 1400-fold more specific on a molar basis for thrombin-antithrombin complex than for antithrombin or prothrombin, respectively. (b) Adsorption-elution of these fractions on columns of thrombin-antithrombin bound to Sepharose 4B generated samples that were ~100- to ~5000-fold more specific on a molar basis for the enzyme-inhibitor interaction product than for antithrombin or prothrombin, respectively. (c) Filtration of fractions obtained from 0 through columns of antithrombin-Sepharose 4B resulted in antibody preparations that were ~13,000- to ~100,000-fold more specific on a molar basis for the enzyme-inhibitor complex than for prothrombin or antithrombin, respectively.

We have also analyzed sera drawn from four rabbits injected with thrombin-antithrombin complex in which the remaining initial reactants had not been eliminated by ion exchange chromatography. Various bleedings were processed as described above to isolate specific antibody populations directed against the enzyme-inhibitor complex. In all cases, the fractions obtained exhibited decreased specificity and were only ~100- to ~5000-fold more reactive on a molar basis with thrombin-antithrombin complex as compared to prothrombin or antithrombin, respectively. This result may be due to the presence of free enzyme or inhibitor within antigen prepared by the latter procedure. These species could induce the formation of antibody populations which are difficult to remove completely from the final product via the affinity chromatographic techniques outlined above.

A radioimmunoassay was constructed that utilized antibody fractions derived from R27, R30, or G184. The three animals had been immunized with thrombin-antithrombin complex prepared by DEAE-cellulose chromatography and the respective products were isolated via affinity chromatographic procedures as described above. These populations of antibodies could immunoprecipitate 85 to 90% of the radiolabeled thrombin-antithrombin complex when employed at a final antibody concentration of ~6.6 µg/ml. However, we usually diluted these products to 0.019 µg/ml in order to maximize the sensitivity of the procedure. Under these conditions, 33% to 50% of the 125I-thrombin-antithrombin counts were immunoprecipitable.

Fig. 2 shows a typical titration curve conducted with antibody fractions derived from R30. The 125I-thrombin-antithrombin bound to the specific antibody in the presence of a given amount of competing antigen divided by the 125I-thrombin-antithrombin bound to the specific antibody in the absence of competing antigen (B/B0) is plotted against the log10 of the molar amount of competing antigen. The solid lines represent a computer-generated fit of the data to the “four parameter” model of Rodbard et al. (44, 45). Results obtained with duplicate assays at varying concentrations of the antibody-thrombin complex demonstrate that the intrasassay coefficient of variation of this method within the linear portion of the titration curve is 3.5%. The detection limit of the technique is 30 ng/ml of thrombin-antithrombin interaction.
Specific Antisera for Thrombin-Antithrombin Complex

The validity of these measurements is dependent upon the stability of the thrombin-antithrombin complex. To demonstrate that the enzyme-inhibitor interaction product does not dissociate during the time required to complete the radioimmunoassay, we have incubated 125I-thrombin-antithrombin complex for 48 h at 4°C with the reagents utilized in this procedure (Second Antibody System I) and have subsequently analyzed the apparent molecular weight of the labeled antigen by SDS-gel electrophoresis. As shown in Fig. 3, the mobility of this material was identical with the 125I-labeled thrombin-antithrombin complex control but distinct from that of either 125I-labeled thrombin or 125I-labeled antithrombin. To prove that trace amounts of proteolytic enzymes within the assay reagents do not partially cleave the enzyme-inhibitor complex and alter its immunoreactivity, we performed the radioimmunoassay utilizing more extensively purified components which had been treated with DFP (Second Antibody System II). Furthermore, we also neutralized any residual levels of thrombin within the radioimmunoassay mixtures by adding the leech protein hirudin at a final concentration of 7.5 × 10⁻⁷ M. As shown in Fig. 2, the immunoreactivity of the thrombin-antithrombin complex is not altered by these precautions.

Throughout this communication, we shall utilize our assay procedure to define the immunoreactivity of a variety of components. Unless otherwise stated, the reactivity of various polypeptides will be estimated by comparing the molar concentrations of a given species to that of the thrombin-antithrombin complex which are required to displace 50% of the immunoprecipitable 125I-thrombin-antithrombin counts from the antibody population.

To examine the relationship between the antigenic site on thrombin-antithrombin complex recognized by our specific antibody population and the three-dimensional conformation of this interaction product, we determined the stability of the immunoreactive region to denaturation as well as reduction and alkylation. To this end, we either heated this species to 100°C or exposed it to 6 M guanidine HCl. Thereafter, thrombin-antithrombin interaction product that had been denatured with guanidine HCl was reduced with DTT and alkylated with 2-iodoacetamide. This latter treatment converts the enzyme-inhibitor complex into a thrombin (heavy chain)-antithrombin interaction product (50). All of the above components were subsequently examined by our specific radioimmunoassay, Fig. 2 and Table I.

Denaturation by heat or guanidine HCl had only a minimal effect upon the immunoreactivity of the thrombin-antithrombin complex and the slope of its logit-log dose-response curve. Reduction and alkylation of the denatured enzyme-inhibitor interaction product drastically reduced the immunoreactivity of this species to an essentially nil value. Thus, our antibody population appears to recognize a specific region within the thrombin-antithrombin complex that is stabilized by one or more S-S bridges.

Immunochemical Studies

Antibody populations derived from R27 and R30 were evaluated to determine their specificity for the thrombin-antithrombin complex. To this end, the immunoreactivity of components or related species which comprise this interaction product as well as other hemostatic enzyme-antithrombin complexes were directly compared to thrombin-antithrombin.

Immunoreactivity of the Thrombin-Antithrombin Complex

Fig. 2. A comparison of antithrombin (O—O), thrombin-antithrombin (●—●), thrombin-antithrombin treated with purified hirudin (X—X), thrombin-antithrombin heated to 100°C for 1 h (□—□), thrombin-antithrombin exposed to 6 M guanidine HCl (▲—▲), and thrombin-antithrombin initially denatured by addition of 6 M guanidine HCL and subsequently alkylated and reduced (C—C). The hirudin-treated complex was added at varying concentrations as competing antigen in a radioimmunoassay utilizing Second Antibody System II where all reagents had been treated extensively with DFP. All other interaction products examined were added at varying concentrations as competing antigens in a radioimmunoassay employing Second Antibody System I. Both assay systems used 125I-thrombin-antithrombin (~0.2 nm) and sufficient antibody from R30 to bind 33% of the radiolabeled ligand. See "Experimental Procedures" and text for additional details.

Fig. 3. SDS-gel electrophoretic analysis of thrombin-antithrombin which has been incubated with the radioimmunoassay reagents or cleaved by the addition of plasmin. a, 125I-thrombin-antithrombin standard; b, 125I-thrombin-antithrombin was mixed with the customary radioimmunoassay reagents including Second Antibody I and incubated for 48 h at 4°C prior to electrophoretic analysis; c, 125I-label thrombin standard; d, 125I-thrombin standard; e, 125I-thrombin-antithrombin incubated with plasmin for 17 h at 4°C prior to electrophoretic analysis. Radiolabeled standards were maintained at 4°C for 48 h in a buffer of 0.1 M sodium phosphate, pH 7.6, that contained 1.0% (w/v) of bovine serum albumin. All samples were adjusted to the same approximate concentration of unlabeled protein prior to electrophoresis. After completion of the separation, gels were sliced into 1-mm segments and the content of 125I was quantitated. Direction of migration is from left (cathode) to right (anode) with the position of the tracking dye indicated by an arrow. See "Experimental Procedures" and text for additional details.
### Specific Antisera for Thrombin-Antithrombin Complex

#### Table 1

<table>
<thead>
<tr>
<th>Component</th>
<th>Component immunoactivity compared to thrombin-antithrombin on a molar basis</th>
<th>Slope of logit-log dose-response curve</th>
<th>No. preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin-antithrombin</td>
<td>1</td>
<td>-1.02</td>
<td>44</td>
</tr>
<tr>
<td>Denatured by heating$^a$</td>
<td>1</td>
<td>-1.05</td>
<td>3</td>
</tr>
<tr>
<td>Denatured by exposure to guanidine HCl$^b$</td>
<td>1</td>
<td>-1.00</td>
<td>3</td>
</tr>
<tr>
<td>Denatured by exposure to guanidine HCl followed by reduction and alkylation</td>
<td>$&gt;20,000$</td>
<td>-0.21</td>
<td>2</td>
</tr>
<tr>
<td>Antithrombin</td>
<td>Not detectable</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Prothrombin</td>
<td>14,000 ± 3,000</td>
<td>-0.60</td>
<td>22</td>
</tr>
<tr>
<td>Pr1</td>
<td>3,100 ± 1,100</td>
<td>-0.49</td>
<td>4</td>
</tr>
<tr>
<td>Pr2</td>
<td>11,000</td>
<td>-0.12</td>
<td>2</td>
</tr>
<tr>
<td>Pr2$^c$</td>
<td>550 ± 320</td>
<td>-0.41</td>
<td>4</td>
</tr>
<tr>
<td>Thrombin</td>
<td>6,000 ± 2,100</td>
<td>-0.38</td>
<td>26</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Not detectable</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Factor IXa</td>
<td>Not detectable</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Factor IXa-antithrombin</td>
<td>$&gt;20,000$</td>
<td>-0.38</td>
<td>8</td>
</tr>
<tr>
<td>Factor IXa treated with DFP prior to the addition of equimolar amounts of antithrombin</td>
<td>$&gt;20,000$</td>
<td>-0.42</td>
<td>2</td>
</tr>
<tr>
<td>Factor X</td>
<td>Not detectable</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Factor Xa</td>
<td>14,000</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Factor Xa-antithrombin</td>
<td>320 ± 80</td>
<td>-0.41</td>
<td>9</td>
</tr>
<tr>
<td>Factor Xa treated with hirudin prior to the addition of equimolar amounts of antithrombin</td>
<td>350</td>
<td>-0.40</td>
<td>2</td>
</tr>
<tr>
<td>Low molecular weight Factor XIIa</td>
<td>Not detectable</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Low molecular weight Factor XIIa-antithrombin</td>
<td>Not detectable</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Not detectable</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Plasmin</td>
<td>33</td>
<td>-1.3</td>
<td>4</td>
</tr>
<tr>
<td>Plasmin treated with DFP</td>
<td>Not detectable</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Plasmin exposed to a 50-fold molar excess of soybean trypsin inhibitor</td>
<td>Not detectable</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Plasmin-antithrombin</td>
<td>Not detectable</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Samples were heated to 100°C for 3 min to 1 h.

$^b$ Samples were exposed to 6 mM guanidine HCl for 2 h at 24°C and subsequently dialyzed against 0.15 M NaCl in 0.05 M sodium phosphate, pH 7.5, prior to use.

$^c$ Samples were denatured by contact with guanidine HCl as described in Footnote b, reduced for 17 h at 24°C by addition of DTT to a final concentration of 20 mM, and subsequently alkylated for 3 h at 24°C by the admixture of 2-iodo[14C]acetamide to a final concentration of 21 mM. All preparations were dialyzed extensively against 0.15 M NaCl in 0.05 M sodium phosphate, pH 7.5, prior to use. The above treatment resulted in the incorporation of ~14 mol of carboxymethyl groups/mol of thrombin-antithrombin complex. Given the known structure of this interaction product, this finding demonstrates that the enzyme-inhibitor complex had been fully reduced and alkylated.

$^d$ The immunoreactivity of these species has been calculated by utilizing an 80% point on the logit-log dose-response curve.

---

**plex, Its Components and Related Species**—Utilizing an antibody population derived from R30, we initially examined the immunoreactivity of antithrombin, which constitutes a major part of the structure of the enzyme-inhibitor complex. As shown in Fig. 2 and Table I, this species reacts minimally with our specific antibody population.

We have also studied the immunoreactivity of prothrombin, activation intermediates such as Pr1, Pr$^*$1, or Pr2, and thrombin. Fig. 4 depicts the results of a typical experiment employing antisera derived from R30 in which the reactivities of these components are directly contrasted with that of the thrombin-antithrombin complex. Table I summarizes data obtained for several preparations of these various molecular species. It is evident that the reactivity of prothrombin is minimal whereas those of the activation intermediates and thrombin are somewhat more significant.

The minimal immunoreactivities of prothrombin and other species derived from this zymogen appear to represent an inherent property of these various components. Firstly, treatment of thrombin with 2.5 mM DFP did not reduce the apparent reactivity of this serine protease. Thus, cleavage of the $^{125}$I-labeled thrombin-antithrombin complex by free enzyme within the radioimmunoassay is not responsible for a spurious, apparent immunoreactivity of this species (see plasmin for comparison).

Secondly, the slopes of the logit-log dose-response curves for prothrombin, activation intermediates, and thrombin are statistically different when compared to that of the enzyme-inhibitor interaction product (Table I). This suggests that the observed cross-reactivities cannot be due to trace contamination of these various preparations with thrombin-antithrombin complex.

Thirdly, we have cross-linked thrombin with dimethyl suberimidate so that its average molecular size is similar to that

---

![Fig. 4. A comparison of thrombin-antithrombin, prothrombin, Pr1, Pr$^*$1, Pr2, and thrombin by radioimmunoassay. Thrombin-antithrombin (●—●), prothrombin (○—○), Pr1 (△—△), Pr$^*$1 (▲—▲), Pr2 (□—□), and thrombin (■—■) were added at varying concentrations as competing antigens in a radioimmunoassay utilizing Second Antibody System I. This procedure employed $^{125}$I-thrombin-antithrombin (~0.2 nM) and sufficient antibody from R30 to bind 33% of the radiolabeled ligand. See "Experimental Procedures" and text for additional details.](image-url)
of the thrombin-antithrombin complex (51) and have noted absolutely no change in its immunoreactivity as compared to monomeric enzyme. Thus, reductions in the antigen valence of the above species secondary to their decreased size via a decrease in the thrombin-antithrombin complex are not responsible for the observed immunoreactivity.

Attempts were made to remove these minimally cross-reacting antibody populations by filtering 15 mg of our material through columns of either prothrombin-Sepharose (0.8 × 13 cm), or thrombin-Sepharose (0.8 × 13 cm), equilibrated in 0.1 M NaCl and 0.01 M sodium azide in 0.05 M potassium phosphate, pH 7.5. Under the conditions of chromatography utilized, ~40% of the specific IgG fraction was bound to either affinity matrix as verified by absorbance measurement or radioimmunoassay. Species which were present in the column effluent did not exhibit improved characteristics with respect to cross-reactivity. Data identical with those cited above were obtained with specific antibody populations derived from R27 or G184 antisera.

**Immunoreactivity of Other Hemostatic System Enzyme Complexes**—Factor IXa, Factor Xa, Factor XIIa, and plasmin are serine proteases known to be neutralized by antithrombin via formation of 1:1 complexes with this inhibitor (21, 22, 24). Moreover, structural homologies have been reported for thrombin, Factor IXa, and Factor Xa (50, 52, 53). For this reason, we investigated the cross-reactivity of our specific antibody population with these protein species as well as with the various complexes which are formed when these enzymes combine with antithrombin.

Factor IX as well as Factor IXa were prepared as outlined under "Experimental Procedures." The final products were analyzed by the radioimmunoassay technique and exhibited no detectable immunoreactivity as compared to the thrombin-antithrombin complex, Fig. 5. The Factor IXa-antithrombin complex was prepared by employing molar ratios of enzyme to inhibitor that varied between 0.70 and 1.10. The environmental conditions utilized were 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5. After admixture of the proteins, heparin was added at a final concentration of 1 × 10⁻⁶ M in order to facilitate complex formation and the solutions were incubated for 50 min at 24°C. Samples that exhibited no enzymatic activity when assayed for Factor IXa were analyzed by SDS-gel electrophoresis. Mixtures were selected that contained a small excess of inhibitor. This allowed us to avoid secondary proteolysis of the Factor IXa-antithrombin interaction product by traces of free enzyme during the radioimmunoassay procedure. Factor IXa is known to form a 1:1 stoichiometric complex with antithrombin. Therefore, we were able to estimate accurately the concentrations of enzyme-inhibitor complex within a reaction mixture based upon the initial level of added Factor IXa. As documented in experiments cited earlier in this communication, the reactivity of antithrombin is essentially nil. Thus, a small excess of inhibitor should have no effect upon the measurement of interaction product immunoreactivity. The inset to Fig. 5 shows an electrophoretic analysis of the final Factor IXa-antithrombin product selected for immunochemoexamination. When this material was studied via the radioimmunoassay procedure utilizing our specific antibody population derived from R30 antisera, we noted that the Factor IXa-antithrombin complex was considerably less immunoreactive than the thrombin-antithrombin complex, Fig. 5. Table I summarizes data obtained for other preparations of this enzyme-inhibitor interaction product. Similar data have been obtained with specific antibody populations derived from R27 or G184 antisera.

The minimal reactivity of the Factor IXa-antithrombin complex appears to represent an intrinsic property of this interaction product. It is not due to trace contamination of Factor IXa preparations with thrombin that subsequently forms enzyme-inhibitor complexes when antithrombin is added. This conclusion is suggested by a statistically significant difference in the slope of the logit-log dose-response curves for Factor IXa-antithrombin complex as compared to that of the thrombin-antithrombin interaction product (Table I). Our interpretation is further bulwarked by treating Factor IXa products with 5 mM DFP prior to forming enzyme-inhibitor complexes as described above. This chemical agent alkylphosphorylates the serine residue of thrombin and prevents it from combining with antithrombin. Factor IXa is not affected by this procedure (54). We noted that the resultant Factor IXa-antithrombin interaction products exhibited no significant decrease in immunoreactivity when compared to those generated without previous exposure of the serine protease to DFP (Table I). Control mixtures were also constructed in which thrombin was treated with 5 mM DFP prior to the addition of equimolar levels of antithrombin. As expected, no significant immunochemochemical signal was detected in these solutions as judged by radioimmunoassay (not shown).

Factor X as well as Factor Xa were purified as described under "Experimental Procedures" and subsequently examined by the radioimmunoassay technique. The zymogen exhibited no detectable immunoreactivity when compared to the thrombin-antithrombin complex, whereas the enzyme generated from this component possessed a small amount of reactivity as contrasted with the thrombin-antithrombin interaction product, Fig. 6. Table I provides data obtained for other preparations of this serine protease.

The Factor Xa-antithrombin complex was formed by utilizing molar ratios of enzyme to inhibitor that varied between 0.3 and 1.1. The environmental conditions used were 0.15 M NaCl in 0.01 M Tris-HCl, pH 7.5. After admixture of the proteins, heparin was added at a final concentration of 1 × 10⁻⁶ M to facilitate complex formation and the respective solutions were incubated for 50 min at 24°C. This enzyme-

![Fig. 5. A comparison of thrombin-antithrombin, Factor IX, Factor IXa, Factor IXa-antithrombin, and Factor IXa that had been treated with DFP prior to addition of equimolar amounts of antithrombin. Thrombin-antithrombin (■—■), Factor XI (○—○), Factor IXa (△—△), Factor IXa-antithrombin (■—■), Factor IXa treated with 5 mM DFP for 1 h at 24°C prior to forming a complex with equimolar amounts of antithrombin (■—■), were added at varying concentrations as competing antigens in a radioimmunoassay which utilized the second Antibody System I. The procedure employed 125I-thrombin-antithrombin (~0.2 nm) and sufficient antibody from R30 to bind 33% of the radiolabeled ligand. See "Experimental Procedures" and text for additional details. Inset, a comparison of antithrombin, Factor IXa, and Factor IXa-antithrombin by SDS-gel electrophoresis. a, Factor IXa; b, antithrombin; c, Factor IXa-antithrombin. Approximately 10 μg of proteins were employed in each electrophoretic examination. Direction of migration is from top (cathode) to bottom (anode).](image)
inhibitor interaction product is not sufficiently stable to be detected by SDS-gel electrophoresis (55). Therefore, mixtures that exhibited no Factor Xa activity were examined by disc gel electrophoretic analysis. Samples that contained a small excess of inhibitor were chosen in order to avoid secondary proteolysis of the Factor Xa-antithrombin interaction product during the subsequent radioimmunoassay procedure. Estimates of the concentration of the enzyme-inhibitor complex within a reaction mixture were obtained as described for the Factor IXa-antithrombin interaction product. The inset to Fig. 6 shows the electrophoretic pattern of a typical Factor Xa-antithrombin preparation selected for immunochemical examination. As expected, small amounts of residual antithrombin and inactive Factor Xa are observed.

When these preparations were studied by radioimmunoassay employing a specific antibody population derived from R30 antisera, we noted that the Factor Xa-antithrombin complex was considerably more reactive than the Factor IXa-antithrombin complex as compared to the thrombin-antithrombin interaction product. In contrast to the thrombin-antithrombin interaction product, the thrombin-antithrombin complex exhibited essentially no immunoreactivity when compared to the thrombin-antithrombin complex when analyzed by radioimmunoassay (Table I). Our specific radioimmunoassay procedure was utilized to analyze their reactivities. When the low molecular weight thrombin-antithrombin complex was analyzed by the radioimmunoassay procedure utilizing a specific antibody population derived from R30 antisera, we observed that this interaction product exhibited essentially no immunoreactivity (Table I). Similar results were noted when the high molecular weight form of Factor XIa was used to generate enzyme-inhibitor interaction products or when specific antibody populations derived from other antisera were employed to monitor the reactivity of this species (not shown).

Plasminogen was isolated as described under "Experimental Procedures." Plasmin was generated from the parent zymogen as previously described (21). The serine protease initially exhibited a significant degree of immunoreactivity when contrasted with the thrombin-antithrombin interaction product (Table I). However, incubation of 50 ng of 125I-radiolabeled thrombin-antithrombin complex with 150 ng of plasmin revealed that extensive cleavage of the enzyme-inhibitor interaction product had taken place (Fig. 3). Therefore, we suspected that the immunoreactivity of plasmin was artifactual and was secondary to proteolytic cleavage of the radiolabeled complex which reduced its precipitability by the specific antibody population. For this reason, we treated plasmin with either 10 mM DFP, or with a 50-fold molar excess of soybean trypsin inhibitor, and re-examined the reactivity of this species by radioimmunoassay. As shown in Table I, neither form of plasmin exhibited any detectable immunoreactivity when compared to the thrombin-antithrombin complex. We have also prepared plasmin-antithrombin interaction product in a manner virtually identical with that utilized in forming other hemostatic enzyme-antithrombin complexes. The plasmin-antithrombin interaction product exhibited no detectable immunoreactivity when compared to thrombin-antithrombin complex by the radioimmunoassay technique (Table I). Similar observations have been made with specific antibody population derived from other antisera (not shown).

Evaluation of the Kinetics of Thrombin-Antithrombin Interaction in Plasma

Our specific radioimmunoassay procedure was utilized to evaluate the importance of antithrombin as a plasma inhibitor
Specific Antiserum for Thrombin-Antithrombin Complex

The radioimmunoassay also permitted us to show that antithrombin, which forms a major portion of the structure of this enzyme-inhibitor interaction product, exhibits essentially no immunoreactivity. Furthermore, prothrombin or species related to this zymogen possess a minimal amount of reactivity. Given the observed immunoreactivity of prothrombin, as well as its concentration within the blood, we may be certain that this component will not affect the specificity of our assay for thrombin-antithrombin complex. Furthermore, the relatively insignificant levels of prothrombin activation intermediates or free thrombin (if it exists) within the blood should not contribute to our assay results despite their slightly higher immunoreactivities.

We have also compared the reactivities of other hemostatic enzyme-antithrombin complexes to that of the thrombin-antithrombin interaction product. The Factor Xa-antithrombin complex is the only component that exhibited any significant degree of immunoreactivity. Its reactivity on a molar basis is approximately 0.19 nM (not shown). Therefore, prothrombin should alter levels of thrombin-antithrombin complex. Given the relatively low levels of Factor Xa in plasma that are generated during the coagulation of blood, the minimal reactivity of the Factor Xa-antithrombin com-

1 The concentration of prothrombin in normal plasma is ~5.5 µM. Given that this zymogen generates a signal which is ~13,000 times less than the thrombin-antithrombin complex on a molar basis, it should contribute no more than ~0.19 µM to the apparent level of enzyme-inhibitor interaction product. The concentration of this latter species within normal plasma is ~2.3 µM (not shown). Therefore, prothrombin should alter levels of thrombin-antithrombin complex by not more than ~8%. In fact, due to the reduced slope of the radioimmunoassay titration for zymogen respectively. The data that less than 3% of the enzyme-inhibitor interaction product signal is caused by the plasma's content of prothrombin. The concentration of activation intermediates within normal plasma is unknown. However, for blood to remain fluid, only 1% or less of the zymogen could be proteolyzed by Factor Xa under in vivo conditions. All of these species exhibit intermediate and much less than the thrombin-antithrombin complex on a molar basis. Thus, these components should contribute to a far smaller extent than prothrombin in our radioimmunoassay.

...
plex should not reduce the specificity of our assay for thrombin-antithrombin complex.

As an example of the potential utility of our assay, we studied the inhibition of thrombin within citrated plasma at a final enzyme concentration of $2.8 \times 10^{-4}$ M. The data revealed that virtually all of the exogenously added enzyme was neutralized via complex formation with antithrombin. These experiments suggest that this protease inhibitor must represent the principle antagonist of thrombin under conditions approaching the in vivo situation. Prior claims that other species may play a primary role in this process when low concentrations of this enzyme are generated can be dismissed. However, the rate of thrombin-antithrombin complex formation within plasma is $\frac{1}{2}$-fold slower than with purified components at equivalent concentrations. These phenomena may be due to the interactions of thrombin with its multiple substrates (1–5) or with other species that retard the inhibitory process (56, 57). Our assay procedure should prove ideally suited for examining biologic modulators which control the rates of thrombin-antithrombin complex formation. This would include the presence of heparin-like molecules that may be liberated in trace amounts into the blood from cellular or vascular sites (58).

REFERENCES