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Inhibition of Bovine Factor IX_a and Factor $X_{a\beta}$ by Antithrombin III†

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ABSTRACT: Factor IX_a and factor $X_{a\beta}$ are serine proteases which participate in the middle phase of blood coagulation. These two enzymes are inhibited by antithrombin III by the formation of an enzyme–inhibitor complex containing 1 mol of enzyme and 1 mol of antithrombin III. The complex was readily demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and loss of coagulant or esterase activity at increasing concentrations of inhibitor. The inactivation of factor IX_a by antithrombin III was relatively slow, but the reaction was greatly accelerated by the addition of heparin.

Antithrombin III is a plasma protein which blocks the enzymatic activity of a number of plasma and pancreatic serine proteases. It inhibits several coagulation factors including thrombin, factor X_a, factor XI_a (Monkhouse et al., 1955; Waugh and Fitzgerald, 1956; Abildgaard, 1968; Biggs et al., 1970; Yin et al., 1971; Dombrose et al., 1971; Damus et al., 1973; Walsh et al., 1974; Highsmith and Rosenberg, 1974), as well as trypsin and chymotrypsin (Abildgaard and Egeberg, 1968).Antithrombin III accounts for the major portion of the antithrombin activity in human plasma as shown by immunoprecipitation experiments (Abildgaard et al., 1970; Rosenberg, 1974). Thus, it is probable that it plays an important physiological role in arresting the coagulation process which is triggered at a site of injury.

Abildgaard (1969) showed by gel filtration that thrombin and antithrombin III form an inactive complex. More recently, Rosenberg and Damus (1973) and Highsmith and Rosenberg (1974) reported that the inhibition of thrombin as well as plasmin by antithrombin III was due to the formation of a stable complex between the inhibitor and enzyme. These investigators employed sodium dodecyl sulfate polyacrylamide gel electrophoresis and identified a new slow moving component corresponding to a 1:1 molar complex of the enzyme and inhibitor. In this manuscript, we wish to report the formation of a similar 1:1 enzyme–inhibitor complex between antithrombin III and factor IX_a and factor $X_{a\beta}$ using similar techniques.

Experimental Section

Materials

Bovine factors IX and X were prepared according to Fujikawa et al. (1972, 1973). Factor $X_{a\beta}$ was prepared as described by Kurachi et al. (1976). Bovine antithrombin III was prepared by method B of Thaler and Schmer (1975). Bovine factor IX_a was kindly provided by P. A. Lindquist in our laboratory. This preparation migrated as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis and has been characterized in previous studies from our laboratory (Fujikawa et al., 1974a). Bovine serum albumin, ovalbumin, carbonic anhydrase, cephalin (rabbit brain extract), and diisopropyl phosphorofluoridate (Dip-F)[1] were purchased from Sigma Chemical Co., St. Louis, Mo. Phosphorylase *b* was a kind donation of Dr. E. H. Fischer. Sephadex G-100 was a product of Pharmacia Fine Chemicals, Piscataway, N.J. 2-Mercaptoethanol and *N,N,N′,N′*-tetramethylethylenediamine were obtained from Eastman Kodak Co., Rochester, N.Y. Acrylamide was obtained from Matheson Coleman and Bell, Norwood, Ohio. Sodium dodecyl sulfate was obtained from British Drug House, Poole, England. Phosphatidylcholine and phosphatidylserine were purchased from Applied Science Laboratories, Inc., Inglewood, Calif. Bovine factor X deficient plasma was made by the method of Bachmann et al. (1958). Benzoylarginyl ethyl ester labeled with [^{3}H]ethanol was kindly provided by E. Fodor of this department. The toluene scintillant was prepared by dissolving 15 g of Omnifluor in 3.8 l. of toluene. Sodium heparin (20 000 units/ml) was purchased from Invenex, San Francisco, Calif. All other chemicals were commercial preparations of the highest quality available.

Methods

Factor X concentration was determined by absorption at 280 nm employing an $E_{280}(1\%)$ of 11.5 (Fujikawa et al., 1974b), and factor $X_{a\beta}$ concentration was determined assuming an $E_{280}(1\%)$ of 10. Factor IX and factor IX_a concentrations were determined by absorption at 280 nm employing an $E_{280}(1\%)$ of 14.9 for factor IX and 14.3 for factor IX_a (Fujikawa et al., 1974b). Bovine antithrombin

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[1] Abbreviation used is: Dip-F, diisopropyl phosphorofluoridate.

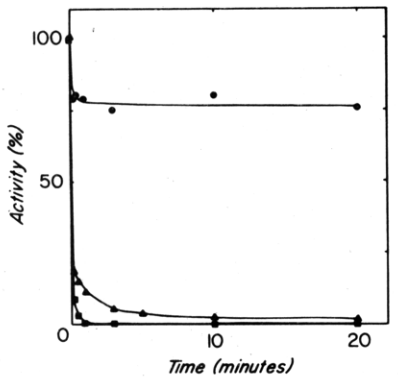

FIGURE 1: Inhibition of bovine factor $X_{a\beta}$ with antithrombin III. The reaction mixture in a total volume of 0.2 ml contained 35×10^{-6} *M* factor $X_{a\beta}$ and 8.9×10^{-6} *M* antithrombin III (●), 10.9×10^{-6} *M* factor $X_{a\beta}$ and 8.9×10^{-6} *M* antithrombin III (▲), and 10.6×10^{-6} *M* factor $X_{a\beta}$ and 35.6×10^{-6} *M* antithrombin III (■) in 0.025 *M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl. At various times of incubation, aliquots (10 μl) were removed and diluted in Michaelis buffer and assayed for factor $X_{a\beta}$ clotting activity as described in the Experimental Section.

III concentration was determined by absorption at 280 nm employing an $E_{280}(1\%)$ of 6.0 (Kurachi et al., 1976).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by a modification of the method of Weber and Osborn (1969) employing 7.5% gels. Samples were run at 7 mA/tube for about 2.5 hr, and the gels were stained for protein with Coomassie Brilliant Blue R. The buffer solution for the electrophoresis was 0.1 *M* Tris-phosphate containing 0.1% sodium dodecyl sulfate (pH 7.0).

Clotting assay for factor $X_{a\beta}$ activity was performed as follows. The test sample was diluted with Michaelis buffer (3.6×10^{-2} *M* sodium acetate–3.6×10^{-2} *M* sodium barbital (pH 7.4)–1.45×10^{-1} *M* sodium chloride) containing 0.1 mg/ml of bovine serum albumin. The sample (0.05 ml of an appropriate dilution) was incubated at 37° for 30 sec with 0.05 ml of phospholipid (one vial of rabbit brain extract suspended in 100 ml of 0.15 *M* NaCl) and with 0.05 ml factor X deficient plasma. A 0.05-ml solution of 0.025 *M* $CaCl_2$ was then added to the incubation mixture and the clotting time was determined. Experiments were run in duplicate and these values agreed within 5%. The percent activity was calculated from a standard curve prepared from factor $X_{a\beta}$.

Esterase assays of factor IX_a and factor $X_{a\beta}$ were performed using ^3H-labeled benzoylarginine ethyl ester according to Roffman et al. (1970) and Anderson et al. (1975). The reaction mixture (10 μl containing about 5 μg or less of enzyme) was transferred into 80 μl of 0.1 *M* Tris-HCl (pH 8.0) buffer solution in a counting vial; 20 μl of 1 m*M* [^3H]benzoylarginyl ethyl ester was then added and mixed, and 10 ml of Omnifluor scintillation liquid was added. Counting was started immediately in a Beckman Model LS-100C scintillation counter at room temperature and the initial rate of hydrolysis of [^3H]benzoylarginyl ethyl ester was recorded. Ester hydrolysis in the presence of buffer and antithrombin III was measured as a blank and subtracted from the initial rate of the test samples. This usually amounted to about 3% or less of the hydrolysis found in the presence of enzyme. In this assay, the rate of hydrolysis of the ester substrate was proportional to the factor IX_a concentration. All measurements were run in duplicate and these values agree within 10%.

Inhibition of Coagulation Factors with Antithrombin III. The inhibition of factor IX_a or factor $X_{a\beta}$ by antithrombin III was carried out by mixing the enzyme and antithrombin III at a concentration of about 0.1% in 0.025 *M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl. The mixture was incubated at 37° for 5 min for factor $X_{a\beta}$ and 2.5 hr for factor IX_a. Preliminary experiments showed that inhibition of factor $X_{a\beta}$ was complete within 5 min, while factor IX_a took at least 2 hr to reach equilibrium with antithrombin III.

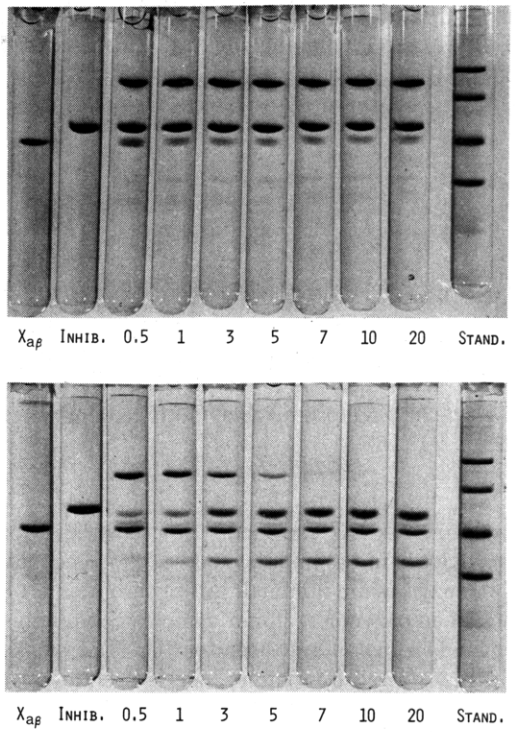

FIGURE 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of factor $X_{a\beta}$ inhibition with antithrombin III. The upper panel corresponds to the inhibition of factor $X_{a\beta}$ in the presence of an excess of antithrombin III (molar ratio 3.4:1) as described in Figure 1. The lower panel corresponds to the inhibition of factor $X_{a\beta}$ in the presence of an excess of factor $X_{a\beta}$ (molar ratio 3.6:1) as described in Figure 1. Aliquots (10 μl) of the reaction mixture containing 20 μg of protein were removed at various times of incubation, and 1 μl of 1 *M* Dip-F and 10 μl of 0.1 *M* Tris-phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate were added. The samples were then heated for 1 min at 100° and applied to the 7.5% polyacrylamide gels. Electrophoresis and staining of the proteins were carried out as described in the Experimental Section. Protein standards are shown in the tubes on the right and include from top to bottom: phosphorylase *b* (95 000), bonic anhydrase (29 000). The numbers under each panel refer to the incubation time in minutes.

Results

Inhibition of Factor $X_{a\beta}$ with Antithrombin III. The time curves for the inhibition of bovine factor $X_{a\beta}$ by bovine antithrombin III are shown in Figure 1. In these experiments, the molar ratio of factor $X_{a\beta}$ to antithrombin III was 3.6:1 (top curve), 1.3:1 (middle curve), and 1:3.4 (bottom curve). When the molar ratio of antithrombin III to factor $X_{a\beta}$ was about 1:1 or greater, essentially 100% inhibition of factor $X_{a\beta}$ activity was observed. Under the conditions of these experiments, the time required for completion of the reaction was only a few minutes. The sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of the proteins in these reaction mixtures are shown in Figure 2. The experiment corresponding to a 3.6:1 molar excess of antithrombin III is shown in the upper panel, and the experi-

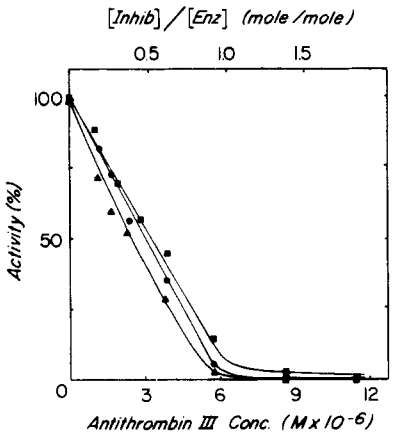

FIGURE 3: Inhibition of factor $X_{a\beta}$ as a function of antithrombin III concentration. The reaction mixture contained 6.3×10^{-6} *M* factor $X_{a\beta}$ in 0.025 *M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl, and increasing concentrations of antithrombin III. The total volume was 0.15 ml. After 5 min of incubation at 38°, aliquots (10 μl) were removed and diluted with Michaelis buffer and assayed for coagulant (●) or esterase (■) activity. Experiments with 20 units of heparin (▲) contained the same amount of factor $X_{a\beta}$, antithrombin III, and buffer solution as described above and aliquots were assayed for coagulant activity.

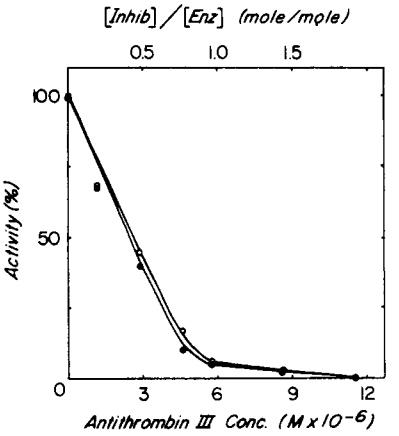

FIGURE 4: Inhibition of factor IX_a esterase activity as a function of antithrombin III concentration. The inhibition mixture contained 6.1×10^{-6} *M* factor IX_a in 0.025 *M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl and various concentrations of antithrombin III. The total volume was 0.05 ml. After 2.5 hr of incubation at 38°, aliquots (20 μl) were removed at various incubation times and assayed for esterase activity. (○) Reaction mixture containing 80 units of heparin; (●) reaction mixture in the absence of heparin.

ment with a 3.4:1 molar excess of factor $X_{a\beta}$ is shown in the lower panel. In each case, the inhibition of factor $X_{a\beta}$ was extremely rapid and led to the formation of a complex with a slower electrophoretic mobility (molecular weight approximately 82 000). This suggests that a very stable complex is formed, and this complex is composed of 1 mol of factor $X_{a\beta}$ (molecular weight 42 000) and 1 mol of antithrombin III (molecular weight 56 000). With an excess of factor $X_{a\beta}$, the complex of enzyme and antithrombin III decomposed as the incubation proceeded, and this gave rise to a smaller fragment with a molecular weight of approximately 35 000 (lower panel, Figure 2). Another major band also appeared at about the same position as intact antithrombin III. Whether this band is partially degraded antithrombin III or some other fragment from the original complex has not been established. It is not native antithrombin III since it does not reform a complex with the factor $X_{a\beta}$ which is still present. These experiments indicate that an excess of antithrombin III yields a stable complex of enzyme and inhibitor, while an excess of enzyme yields a complex which is subsequently degraded. This suggests that the degradation of the complex in the latter experiment was due to proteolysis by factor $X_{a\beta}$.

The inhibition of factor $X_{a\beta}$ as a function of antithrombin III concentration is shown in Figure 3. Factor $X_{a\beta}$ and antithrombin III in the presence or absence of heparin were incubated for 5 min at 38° and aliquots were assayed for residual coagulant or esterase activity. Inhibition of factor $X_{a\beta}$ with antithrombin III was essentially the same in the presence or absence of heparin, indicating that heparin has little or no effect on the final equilibrium between the enzyme and inhibitor. Also, the inhibition of esterase activity was essentially identical with the inhibition of coagulant activity, suggesting that the reactive sites for esterase and coagulant activity are identical.

The enzyme concentration in these experiments was 6.3×10^{-6} *M*. Extrapolation of the curves in Figure 3 to zero activity gives an intercept of 5.8×10^{-6}, 6.3×10^{-6}, and 6.7×10^{-6} *M* antithrombin III for the inhibition of factor $X_{a\beta}$ coagulant activity in the presence and absence of heparin, and inhibition of esterase activity, respectively. These experiments provide further evidence for the conclusion that antithrombin III forms an equimolar complex with factor $X_{a\beta}$ in the presence or absence of heparin. The association constant estimated from these experiments ranged from 5×10^7 to 5×10^8 *M*.

In control experiments, it was shown that factor X does not form a stable complex with antithrombin III in the presence or absence of heparin, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Inhibition of Factor IX_a with Antithrombin III. The inhibition of factor IX_a esterase activity with increasing concentration of antithrombin III is shown in Figure 4. These experiments were also carried out in the presence and absence of heparin. Antithrombin III was incubated with factor IX_a at 38° for 2.5 hr to ensure complete equilibration of enzyme and inhibitor.

The inhibition of factor IX_a esterase activity increased with increasing concentration of antithrombin III and the degree of inhibition was the same in the presence or absence of heparin. The association constant calculated from these data was about 2×10^7 *M*.

The concentration of factor IX_a employed in these experiments was 6.1×10^{-6} *M*. Extrapolation of the curves in Figure 4 to zero esterase activity gives an intercept of $5.2-5.4 \times 10^{-6}$ *M* antithrombin III. These experiments indicate that factor IX_a interacts with antithrombin III by forming a 1:1 molar complex. This conclusion was supported by experiments in which factor IX_a was incubated with antithrombin III in the presence or absence of heparin and the products were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 5). The top panel shows a reaction mixture containing heparin, factor IX_a, and an excess of antithrombin III. Within the first minute, all of the factor IX_a is converted to a new slow moving band with a molecular weight of approximately 85 000. This indicates that a very stable complex is formed between the enzyme and inhibitor, and this complex is composed of 1 mol of factor IX_a (molecular weight 46 500) and 1 mol of antithrombin III (molecular weight 56 000).

In the absence of heparin, the complex of enzyme and inhibitor is formed very slowly and can be identified on the

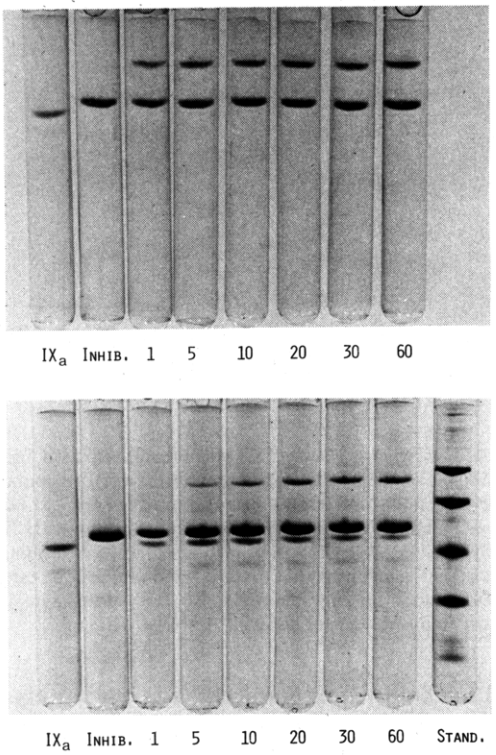

FIGURE 5: Sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of factor IX_a inhibition with antithrombin III. The upper panel corresponds to the inhibition of factor IX_a and antithrombin III in the presence of heparin, and the lower panel corresponds to the inhibition of factor IX_a and antithrombin III in the absence of heparin. The reaction mixtures (0.2 ml) contained 5.5×10^{-6} *M* factor IX_a and 17×10^{-6} *M* antithrombin III. The reaction mixture shown in the upper panel also contained 80 units of heparin. At various times of incubation, aliquots (20 μl) were added to 10 μl of 0.1% sodium dodecyl sulfate Tris-phosphate and 1 μl of 1 *M* Dip-F. The samples were heated for 1 min at 100° and applied to 7.5% polyacrylamide gels. Electrophoresis and staining of the proteins were carried out as described in the Experimental Section. Protein standards are as described under Figure 2. The numbers under each panel refer to the incubation time in minutes.

sodium dodecyl sulfate polyacrylamide gels only after about 5 min of incubation (lower panel, Figure 5). The amount of the complex continues to increase with time and the reaction reaches completion in about 90 min. Thus, factor IX_a is inhibited very slowly by antithrombin III in the absence of heparin. In the presence of heparin, however, the reaction is greatly accelerated and is complete within 1 min of incubation.

In control experiments with factor IX and antithrombin III in the presence or absence of heparin, no stable complex was formed as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Discussion

In the present experiments, the formation of a stable complex between factor IX_a or factor $X_{a\beta}$ and antithrombin III was shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular weight of the complex estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis was 82 000 and 85 000 for antithrombin III and factor $X_{a\beta}$ or factor IX_a, respectively. These values are lower than the summation of equimolar amounts of each of the two enzymes and inhibitor (approximately 100 000). Proteolysis of the complex between factor $X_{a\beta}$ and antithrombin III occurs in the presence of excess enzyme and degradation products are observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (lower panel, Figure 2). Very little low molecular weight degradation fragments are observed with excess antithrombin III and factor $X_{a\beta}$ (upper panel, Figure 2) and factor IX_a and antithrombin III (Figure 5). This suggests that the low estimate for the molecular weight of the complex between the two enzymes and inhibitor is due to an artifact of the sodium dodecyl sulfate polyacrylamide gel electrophoresis technique.

Evidence for a 1:1 complex between enzyme and inhibitor was also obtained from experiments in which the enzymatic activity of the two coagulation factors was examined at increasing concentrations of antithrombin III. The stoichiometry of this complex was not influenced by heparin. This effector only influenced the rate of inactivation of the enzyme, as shown in the case of factor IX_a inactivation by antithrombin III. This suggests that heparin exerts its effect as an allosteric effector on antithrombin III.

The relative rates of inactivation of factor $X_{a\beta}$ and factor IX_a by antithrombin III in the presence of heparin are not known. In the absence of heparin, factor $X_{a\beta}$ is far more sensitive to inhibition by antithrombin III than factor IX_a. In these experiments, the concentrations and ratios of factor IX_a or factor $X_{a\beta}$ to inhibitor were about the same.

In the intrinsic pathway of blood coagulation, factor IX_a converts factor X to factor X_a in the presence of factor VIII, calcium ions, and phospholipid (Fujikawa et al., 1974b). In this reaction, a single specific internal arginyl–isoleucine bond is cleaved. The rapid inactivation of factor X_a by antithrombin III makes it difficult to measure the inhibition of factor IX_a by coagulant activity since these two proteins are so closely associated in the coagulation scheme. This problem was avoided, however, by employing an esterase assay for factor IX_a using benzoylarginyl ethyl ester as substrate.

The nature of the binding sites between factor IX_a or factor $X_{a\beta}$ and antithrombin III is not known. The stability of the complex in the presence of 0.1% sodium dodecyl sulfate (pH 7.0) at 100° suggests that it may involve a covalent bond with the reactive serine of factor IX_a (Enfield et al., 1974) or factor $X_{a\beta}$ (Titani et al., 1972). Indeed, the enzyme–antithrombin III complex may be analogous to the tetrahedral intermediate which has been identified by crystallographic techniques between trypsin and pancreatic trypsin inhibitor (Ruhlmann et al., 1973) and trypsin and soybean trypsin inhibitor (Sweet et al., 1974). In these two cases, a tetrahedral adduct is formed between the active serine of the enzyme and a specific lysine or arginine residue in the inhibitor.

The complex between factor X_a and antithrombin III was degraded in the presence of excess enzyme. The nature of the products from this reaction has not been established. This reaction may be similar to that reported by Iwasaki et al. (1974) for the degradation of the complex between trypsin and a potato protease inhibitor. This inhibitor (molecular weight 10 500) forms a complex with trypsin, and in the presence of excess enzyme the inhibitor is converted into a smaller fragment (molecular weight 4800) which also has potent antitrypsin activity.

The physiological importance of antithrombin III in the inactivation of thrombin appears to be well established (Abildgaard et al., 1970; Rosenberg, 1974). The physiological role of antithrombin III in the inactivation of factor X_a, however, is unclear. Marciniak (1973) has shown that the inhibition of factor X_a by antithrombin III was greatly re-

duced in the presence of factor V, calcium ions, and phospholipid. Thus, factor X_a was not readily inactivated by antithrombin III after complex formation with factor V, calcium ions, and phospholipid occurred. Antithrombin III may, however, compete with factor V for factor X_a, and this reaction may be very important, particularly in the presence of heparin.

Whether factor IX_a is protected from antithrombin III by factor VIII, calcium ions, and phospholipid in a similar manner is not known. Factor IX_a is protected from inactivation by hirudin in the presence of factor VIII, calcium ions, and phospholipid (M. E. Legaz and E. W. Davie, unpublished experiments). Thus, it is possible that factor IX_a is also protected from antithrombin III and heparin in the presence of factor VIII, calcium ions, and phospholipid.

Acknowledgments

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