Detection of a New Heparin-dependent Inhibitor of Thrombin in Human Plasma

DOUGLAS M. TOLLEFSEN and MARY K. BLANK, *Division of Hematology-Oncology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110*

ABSTRACT We have demonstrated that human plasma contains a heparin-dependent inhibitor of thrombin that is distinguishable from antithrombin III (AT III). When a 1:50 dilution of plasma was incubated with ≥0.01 U/ml heparin and 1 U/ml ^{125}I-thrombin, the labeled thrombin B-chains became incorporated into two complexes of M_r-96,000 and M_r-85,000 that were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol. Neither complex was detectable at heparin concentrations <0.01 U/ml. When a limiting amount of ^{125}I-thrombin was present, the proportion of radioactivity incorporated into each of the two complexes varied with the heparin concentration. Thus, the M_r-85,000 complex predominated at 0.01–5 U/ml heparin, whereas the M_r-96,000 complex predominated at 5–100 U/ml heparin. The M_r-85,000 complex reacted with antibodies to human AT III and comigrated with the purified thrombin-AT III complex. The M_r-96,000 complex did not react with antibodies to AT III or to α1-antitrypsin, and it was detected in normal quantities after incubating ^{125}I-thrombin with plasma immunodepleted of AT III, α2-antiplasmin, α2-macroglobulin, $\overline{C1}$ inactivator, α1-antichymotrypsin, or inter-α-trypsin inhibitor. The protein that combines with thrombin to form the M_r-96,000 complex was estimated to be present at a minimum concentration of 90±26 μg/ml (mean±SD) in normal plasma. We conclude that the protein is not identical to any of the known plasma protease inhibitors and that at relatively high heparin concentrations in vitro it reacts with thrombin more rapidly than does AT III.

INTRODUCTION

Heparin has been known for many years to be a potent anticoagulant and is widely used in the treatment of thromboembolic disorders in man (1). It is generally accepted that heparin exerts its anticoagulant effect by activating the plasma protein antithrombin III (AT III)[1] (formerly called "heparin cofactor") (2). AT III inhibits thrombin and other coagulation proteases (Factors Xa, IXa, XIa, XIIa, and kallikrein) by forming 1:1 stoichiometric complexes with each protease (2). That the complexes are stable in denaturing agents suggests that a covalent bond is formed between the protease and the inhibitor and allows detection of the complexes in sodium dodecyl sulfate (SDS) polyacrylamide gels.

It is clear that heparin accelerates the rate at which AT III reacts with various proteases in vitro. In the case of thrombin, the second-order rate constant of complex formation increases from 4.3×10^5 M^{-1} min^{-1} in the absence of heparin to 8×10^8 M^{-1} min^{-1} at optimal heparin concentrations (3). None of the other recognized protease inhibitors in plasma[2] is known to be stimulated by heparin, with the possible exception of $\overline{C1}$ inactivator (5), which does not inhibit thrombin (6). On the other hand, several previous reports have claimed to separate from AT III a thrombin inhibitor with heparin cofactor activity, although the material responsible for this activity was neither purified nor characterized by immunological techniques (7–9). Thus, the existence of a heparin cofactor apart from AT III has remained in doubt.

In the present investigation, we have incubated ^{125}I-labeled thrombin with plasma and separated the

This work was presented in part at the Annual Meeting of the American Society for Clinical Investigation, 25–27 April 1981, San Francisco, and has appeared in abstract form. 1981. *Clin. Res.* **29**: 523A.

Received for publication 9 March 1981 and in revised form 21 April 1981.

[1] *Abbreviations used in this paper*: AT III, antithrombin III; SDS, sodium dodecyl sulfate.

[2] These include α1-antitrypsin, α1-antichymotrypsin, inter-α-trypsin inhibitor, α2-antiplasmin, AT III, $\overline{C1}$ inactivator, and α2-macroglobulin (4).

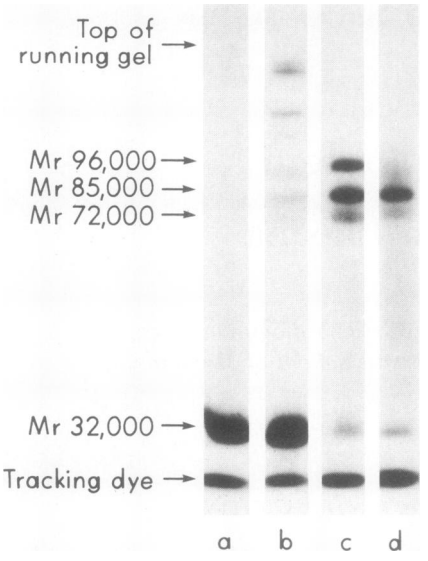

FIGURE 1 Interaction of ^{125}I-thrombin with plasma and purified AT III. Samples contained either plasma (1 μl) or AT III (1 μl of a 200-μg/ml stock solution) in a total volume of 50 μl of 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4. ^{125}I-thrombin and heparin at final concentrations of 1 U/ml and 5 U/ml, respectively, were also present as indicated. After a 10-min incubation at 25°C, each sample was prepared for electrophoresis and autoradiography as described in Methods. The autoradiogram is shown. (*a*) ^{125}I-thrombin, (*b*) ^{125}I-thrombin + plasma, (*c*) ^{125}I-thrombin + plasma + heparin, (*d*) ^{125}I-thrombin + purified AT III + heparin.

resulting inhibitor-protease complexes by SDS polyacrylamide gel electrophoresis. In this manner we have demonstrated the presence of a previously unrecognized thrombin inhibitor with heparin cofactor activity. The new inhibitor is immunologically distinct from AT III and from all of the other known protease inhibitors in plasma. The inhibitor is present in plasma at a concentration somewhat lower than that of AT III. In the presence of relatively high concentrations of heparin, however, thrombin forms complexes preferentially with the new inhibitor.

METHODS

Materials. Blood was drawn from normal volunteers into plastic syringes containing 6 mM EDTA (final concentration). Cells were removed by centrifugation, and the plasma was stored at −20°C before use. Heparin derived from porcine intestinal mucosa (Panheprin, Abbott Diagnostics, Diagnostic Products, North Chicago, Ill., 1,000 U. S. Pharmacopeia U/ml; or Lipo-Hepin, Riker Laboratories, Inc., Northridge, Calif., 20,000 U. S. Pharmacopeia U/ml) was diluted in buffer containing 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.4, immediately before use; the biological activity was assumed to be that stated by the manufacturer. According to the carbazole assay for uronic acid (10), the specific activities of the two heparin preparations were 159 U/mg (Abbott Diagnostics) and 144 U/mg (Riker). Rabbit antisera directed against human AT III, α1-antitrypsin, α1-antichymotrypsin, α2-macroglobulin, $\overline{C1}$ inactivator, and inter-α-trypsin inhibitor were purchased from Calbiochem-Behring Corp., American Hoechst, La Jolla, Calif. Rabbit antiserum directed against human α2-antiplasmin was purchased from Nordic Immunological Laboratories (USA), San Clemente, Calif. Apiezon A oil was obtained from James B. Biddle Co., Plymouth Meeting, Pa.

Proteins. Human prothrombin was prepared by the method of Miletich et al. (11). Prothrombin was activated using Taipan snake venom (12) and thrombin was subsequently isolated by chromatography on Amberlite CG-50 resin (13). Thrombin activity was measured in a fibrinogen-clotting assay (13) standardized with U. S. Standard Thrombin, Lot J, obtained from Dr. D. L. Aronson, Bureau of Biologics, Food and Drug Administration, Bethesda, Md. Protein concentration was determined by ultraviolet absorbance, assuming $E_{280}^{1\%} = 18.3$ for thrombin (13). The specific activity ranged from 2,400 to 2,700 U/mg thrombin. The preparation was homogeneous by SDS polyacrylamide gel electrophoresis, which indicated the absence of detectable quantities of β- and γ-thrombin (13).

Human AT III was purified by the method of Thaler and Schmer (14). In a standard clotting assay, the preparation inhibited 1,500–2,000 U of thrombin/mg of AT III, on the assumption of $E_{280}^{1\%} = 5.7$ for AT III (15). The AT III was homogeneous and had an M_r of 59,000 by SDS polyacrylamide gel electrophoresis.

Iodination of thrombin. Thrombin was iodinated by the chloramine-T method described previously (16), modified to include 20 μM unlabeled NaI in the reaction mixture. This resulted in the incorporation of ~0.5 atom of I/molecule of thrombin, and in a specific radioactivity of $2-4 \times 10^6$ cpm/U thrombin. The ^{125}I-thrombin had >90% of the specific clotting activity of the unlabeled starting material. In parallel incubations containing 10 μg/ml of purified AT III and 0.08 μg/ml of either ^{125}I-thrombin or unlabeled thrombin, the rates of inactivation of the two thrombins were identical (data not shown). The second-order rate constant calculated for the inactivation of ^{125}I-thrombin by AT III was 5.3×10^5 M^{-1} min^{-1}, in agreement with the value for unlabeled thrombin reported by others (3).

SDS gel electrophoresis. Samples were heated at 100°C for 2 min with an equal volume of a solution containing 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.125 M Tris-HCl, pH 6.8. Electrophoresis was performed with slab gels containing 7.5% polyacrylamide in the running gel and 4% polyacrylamide in the stacking gel according to the procedure of Laemmli (17). The gels were fixed and stained with a solution containing 0.25% Coomassie Blue G-250, 10% acetic acid, and 50% methanol. The gels were destained with a solution containing 10% acetic acid and 25% ethanol, and dried with a Hoefer model SE540 slab gel dryer (Hoefer Scientific Instruments, San Francisco, Calif.). Molecular weight standards included myosin (M_r 200,000) β-galactosidase (M_r 116,500), phosphorylase B (M_r 94,000), bovine serum albumin (M_r 68,000), and ovalbumin (M_r 43,000).

Autoradiography. Kodak RP X-Omat film (Eastman Kodak Co., Rochester, N. Y.) was placed between the dried slab gel and a Dupont Cronex Lightning Plus intensifier screen (E. I. Du Pont de Nemours & Co., Wilmington, Del.) for 6–18 h at −70°C (18), and the film was developed according to the manufacturer. Selected bands identified in the autoradiogram were traced onto the dried gel, cut out, and counted in a Beckman model 300 gamma counter (Beckman Instruments, Inc., Fullerton, Calif.).

Immunoabsorption of plasma. Heat-killed, formalin-fixed *Staphylococcus aureus* (Cowan I strain) was provided by Dr. Benjamin Schwartz, Washington University, St. Louis, Mo.

Immediately before use, the bacteria were washed with 0.25% Nonidet P-40 in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, and resuspended in the same buffer without detergent at a concentration of 8–10% (packed cell volume). Bacteria were coated with antibodies specific for one of the plasma protease inhibitors by incubating 0.5 ml of the *S. aureus* suspension with 0.5 ml of rabbit antiserum for 2 h at 25°C. The bacteria were washed in the above buffer (without detergent) to remove unbound serum proteins and then sedimented at 12,000 *g* for 10 min to produce a tightly packed pellet. 50 μl of human plasma was then mixed with the bacterial pellet (~50 μl, packed cell volume) and allowed to incubate for 2 h at 25°C. The bacteria were removed by centrifugation and the absorbed plasma was stored at −20°C. Protein concentrations of the absorbed plasma samples were measured by the Lowry method (19) and found to be ~85% of that of the starting plasma, which indicates a minor degree of dilution of the plasma during the absorption procedure.

Ouchterlony analysis. Double immunodiffusion was performed with 1%-agarose plates prepared by standard methods (20). Diffusion was allowed to proceed for 24–48 h in a humidified chamber at 25°C, and the plates were then photographed. In some cases, the plates were washed, dried, and stained for protein with Coomassie Blue R-250.

Immunoassay of AT III. AT III concentration in plasma was determined by radial immunodiffusion with agar plates containing antisera to human AT III (M-Partigen plates, Calbiochem-Behring Corp.) (21). The assay was standardized with purified AT III.

RESULTS

^{125}I-Thrombin preparation. Thrombin consists of two polypeptide chains linked by a disulfide bond. They include the A-chain (M_r 4,600) and the B-chain (M_r 32,000), the latter containing the active center serine residue of the protease (13). In reduced SDS polyacrylamide gels, the ^{125}I-thrombin used in the experiments to follow gave the pattern shown in Fig. 1a. Most of the radioactivity (73–84% in different preparations) was present as a single band at the position of the B-chain. The remaining 16–27% of the radioactivity migrated with the tracking dye and was assumed to represent the dissociated A-chain.

Heparin-dependent complexes of ^{125}I-thrombin and plasma proteins. When ^{125}I-thrombin was incubated with plasma (diluted 1:50) in the absence of heparin, 20% of the ^{125}I became incorporated into several complexes of M_r 72,000–200,000 in reduced SDS gels (Fig. 1b). Bands representing the uncomplexed A- and B-chains contained 17 and 63% of the ^{125}I, respectively. The complexes observed in this experiment were not characterized further. 7% of the ^{125}I, however, was found in bands of M_r 72,000 and 85,000, which probably represent complexes of ^{125}I-thrombin with AT III, as shown below. This is precisely the extent of complex formation with AT III expected after a 10-min incubation, on the assumption of a second-order rate constant of 2.07×10^3 M^{-1} s^{-1} (22) and a concentration of 4 μg AT III/ml in the incubation mixture.

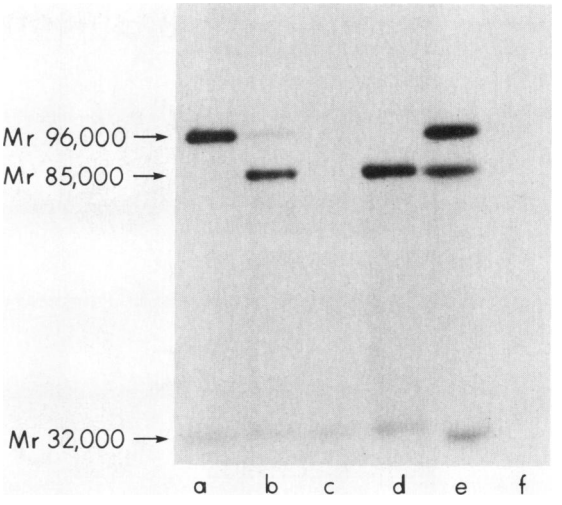

FIGURE 2 Reaction of ^{125}I-thrombin-inhibitor complexes with antiserum to AT III. All incubations were performed at 25°C in buffer containing 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.4. Plasma (5 μl) or purified AT III (5 μl of a 200-μg/ml solution) was incubated with 5 U/ml heparin and 1 U/ml ^{125}I-thrombin in a total volume of 250 μl. The ^{125}I-thrombin was added last, after a 1-min preincubation of the other reactants. After a 10-min incubation, 5 μl of antiserum was added. 30 min later, 50 μl of an 8% (vol/vol) suspension of staphylococci prepared as described in Methods was added, and the tubes were stirred gently for an additional 30 min. The bacteria were removed by centrifugation through 300 μl of 10% Apiezon A oil in *n*-butyl phthalate in an Eppendorf microcentrifuge, and the pellet was resuspended in 300 μl of buffer. Aliquots (50 μl) of the supernate (*a*, *c*, and *e*) and the resuspended pellet (*b*, *d*, and *f*) were prepared for electrophoresis as described in Methods. The autoradiogram is shown. (*a* and *b*) ^{125}I-thrombin + plasma + anti-AT III antiserum, (*c* and *d*) ^{125}I-thrombin + AT III + anti-AT III antiserum, (*e* and *f*) ^{125}I-thrombin + plasma + anti-α1-antitrypsin antiserum.

A different pattern was observed when 5 U/ml heparin was included in the incubation (Fig. 1c). Under these conditions, 50% of the radioactivity was found in three complexes of M_r 72,000, 85,000, and 96,000. An additional 15% of the ^{125}I was distributed uniformly throughout the area of the gel between the M_r-72,000 and M_r-32,000 bands (discussed below). There was a concomitant reduction in the amount of radioactivity at the position of the thrombin B-chain (now only 18% of the total counts per minute), whereas the radioactivity at the tracking dye did not change significantly when compared with the incubation without heparin. Control incubations containing ^{125}I-thrombin and heparin without plasma did not form complexes (not shown). The observed complexes, therefore, appear to result from covalent bond formation between plasma proteins and the B-chain of thrombin.

The M_r-72,000 and M_r-85,000 bands in Fig. 1c contained ~⅔ of the complexed B-chain radioactivity, and they comigrated with the products formed when

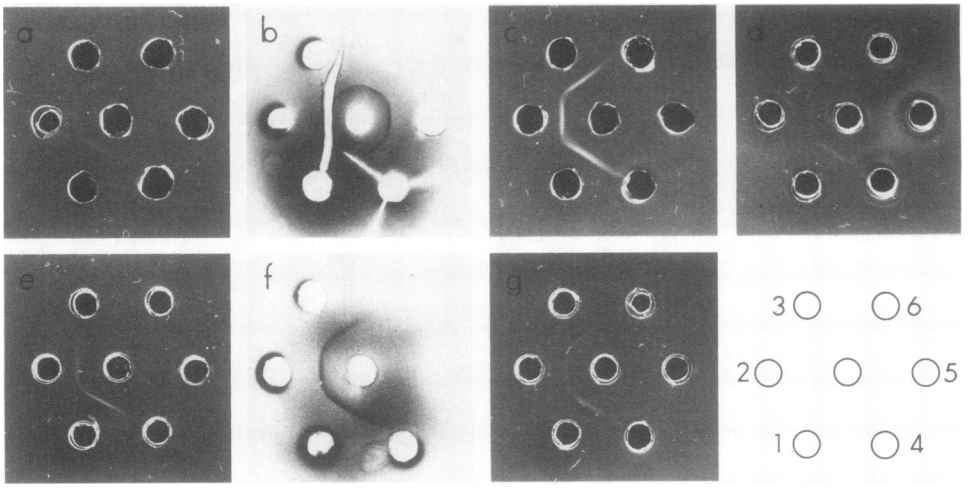

FIGURE 3 Ouchterlony analysis of inhibitor-depleted plasmas. Plasma samples were absorbed with antisera to specific protease inhibitors and immunodiffusion was performed as described in Methods. In each case, the center well contained 5 μl of the antiserum used to absorb the plasma; wells *1*, 2, and *3* contained 5 μl of control unabsorbed plasma (undiluted, 1:2, and 1:4, respectively); and wells *4*, 5, and *6* contained 5 μl of absorbed plasma (undiluted, 1:2, and 1:4). Antisera to the following inhibitors were used: *a*, α2-antiplasmin; *b*, α1-antitrypsin; *c*, α2-macroglobulin; *d*, antithrombin III; *e*, $\overline{C1}$ inactivator; *f*, α1-antichymotrypsin; and *g*, inter-α-trypsin inhibitor. Plates *b* and *f* were dried and stained before being photographed.

^{125}I-thrombin was incubated with an amount of purified AT III equal to that present in the plasma sample (Fig. 1d). The two bands had approximately the same apparent molecular weights as the intact thrombin-AT III complex and the major degradation product of this complex reported by others (23, 24). In subsequent experiments (Figs. 2, 4, and 5) heparin was preincubated with plasma or AT III for 1 min before the addition of ^{125}I-thrombin, and the M_r-72,000 band was no longer seen.

The M_r-96,000 band shown in Fig. 1c contained ~⅓ of the complexed B-chains in the presence of 5 U/ml heparin. The band was virtually undetectable in incubations without heparin (Fig. 1b) and did not form in the reaction of purified AT III and ^{125}I-thrombin (Fig. 1d). Since electrophoresis was carried out in the presence of SDS and β-mercaptoethanol, it is unlikely that the M_r-96,000 band is a degradation product of the M_r-85,000 thrombin-AT III complex. These results suggest that plasma contains in addition to AT III a second protein capable of forming a complex with thrombin in the presence of heparin.

Lack of immunoreactive AT III in the M_r*-96,000 complex.* Alternatively, we considered that the M_r-96,000 band might contain a precursor of AT III or result from interaction of the thrombin-AT III complex with a third plasma component. In either case the complex might have been found (although not necessarily) to contain antigenic determinants in common with native AT III. Fig. 2 demonstrates that this was not the case. Although the M_r-85,000 complex was quantitatively removed from plasma with antiserum to AT III and protein A-bearing staphylococci (25), >90% of the M_r-96,000 complex remained in the supernate (Fig. 2, a and b). Doubling the quantity of antiserum and staphylococci did not result in removal of more of the M_r-96,000 complex (not shown). The M_r-85,000 complex prepared from ^{125}I-thrombin

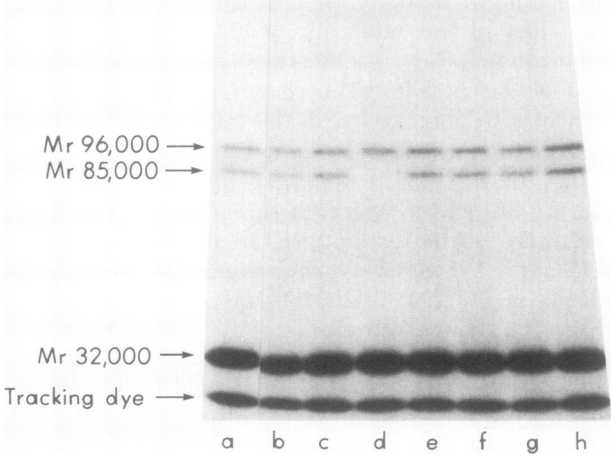

FIGURE 4 Formation of ^{125}I-thrombin-inhibitor complexes in inhibitor-depleted plasmas. Plasma samples were incubated with 10 U/ml ^{125}I-thrombin and 5 U/ml heparin, and electrophoresis was performed as in Fig. 1. The ^{125}I-thrombin was added after a 1-min preincubation of the other reactants. Lanes *a*–*g* of the autoradiogram correspond to the absorbed plasma samples in plates *a*–*g*, respectively, in Fig. 3. Lane *h* represents a control incubation with unabsorbed plasma.

and purified AT III was absorbed as expected under identical conditions (Fig. 2, c and d). When nonimmune rabbit serum or antiserum against human α1-antitrypsin (Fig. 2, e and f) was substituted for anti-AT III antiserum, neither complex was found in the bacterial pellet.

Formation of the M_r*-96,000 complex in plasma samples depleted of known protease inhibitors.* We next absorbed aliquots of plasma with antibodies directed against individual protease inhibitors (for details see Methods), and we assayed each absorbed plasma for the ability to form complexes with ^{125}I-thrombin. The effectiveness of the absorption procedure was first confirmed by double immunodiffusion analysis. Fig. 3 demonstrates that a 1:4 dilution of unabsorbed plasma formed a visible precipitin line when allowed to react with antiserum to each of the inhibitors tested. In contrast, undiluted samples of plasma that had been absorbed with antibodies against α2-antiplasmin, α2-macroglobulin, antithrombin III, $\overline{C1}$ inactivator, α1-antichymotrypsin, and inter-α-trypsin inhibitor failed to form precipitin lines with the absorbing antisera. When the ~15% dilution of the plasma samples during absorption (see Methods) is taken into account, Fig. 3 indicates substantial (i.e., >⅔) depletion of the inhibitors from these plasma samples. α-1-antitrypsin, which is present in plasma at an 8- to 56-fold greater molar concentration than any of the other inhibitors (4), was not effectively removed by this technique (Fig. 3b).

When the absorbed plasma samples were incubated with ^{125}I-thrombin and heparin, the M_r-96,000 complex was formed in each case (Fig. 4, a–g). The M_r-85,000 complex was also present as expected, except in the plasma sample depleted of AT III (Fig. 4d). The radioactivity in the M_r-96,000 band in each of the absorbed plasma samples was 71–93% of that present in unabsorbed plasma (Fig. 4h). It is important to note that a saturating concentration of ^{125}I-thrombin (see below) was used to maximize complex formation in these incubations. With the exception of α1-antitrypsin, for which the data are inconclusive, the experiments in Figs. 3 and 4 indicate that the M_r-96,000 complex does not result from interaction of ^{125}I-thrombin with any of the known protease inhibitors of plasma.

Heparin-dependence of the M_r*-96,000 complex compared with that of the thrombin-AT III complex.* We determined the extent of complex formation when ^{125}I-thrombin was added to plasma, purified AT III, or AT III-depleted plasma in the presence of various concentrations of heparin. When a limiting amount of ^{125}I-thrombin was added to plasma (Fig. 5, a and b), the amount of radioactivity present in the M_r-85,000 thrombin-AT III complex was maximal at 0.01–0.1 U/ml of heparin. At these relatively low heparin

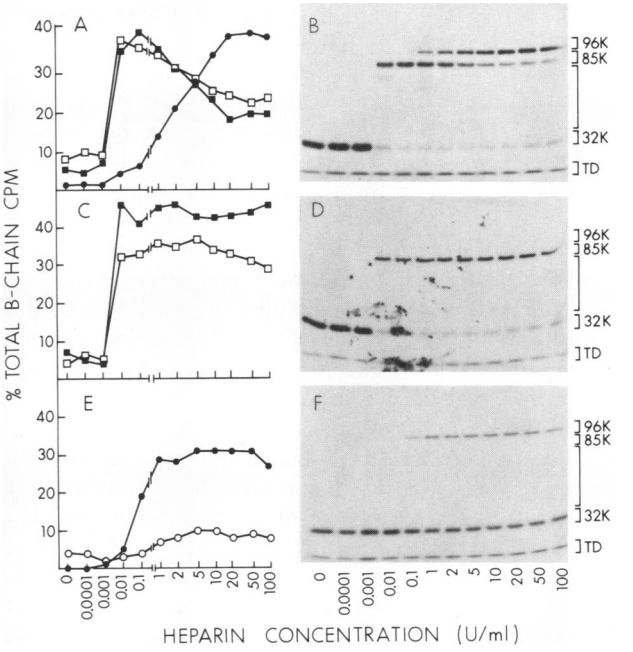

FIGURE 5 Formation of ^{125}I-thrombin-inhibitor complexes at various heparin concentrations. Unabsorbed plasma (*A* and *B*), purified AT III (*C* and *D*), and AT III-depleted plasma (*E* and *F*) were incubated with 1 U/ml ^{125}I-thrombin as in Fig. 1 after a 1-min preincubation with 0–100 U/ml heparin. Electrophoresis and autoradiography were performed as described in Methods. Selected bands identified by autoradiography (bracketed) were cut out of the dried gels and counted for ^{125}I. The total ^{125}I-thrombin B-chain counts per minute was assumed to be the sum of the counts per minute found in bands above the tracking dye (TD). Panels *A*, *C*, and *E* show the percentage of the total B-chain counts per minute found in the following bands: ●, M_r-96,000 complex; ■, M_r-85,000 complex; □, area between the M_r-85,000 and M_r-32,000 bands; ○, area between the M_r-96,000 and M_r-32,000 bands. Panels *B*, *D*, and *F* show the autoradiograms.

concentrations, a faint M_r-96,000 band was also observed. Increasing the concentration of heparin from 0.1 to 20 U/ml resulted in a substantial increase in the amount of radioactivity in the M_r-96,000 band and a concomitant decrease in the intensity of the M_r-85,000 band. At concentrations >20 U/ml, the proportion of ^{125}I counts per minute in the two bands did not change. Identical results were obtained with heparin preparations from two commercial sources (see Methods).

Preliminary experiments in which ^{125}I-thrombin (1 U/ml) was added to whole blood collected directly into syringes containing various amounts of concentrated heparin have yielded qualitatively similar results,[3] although the concentrations of heparin re-

[3] D. M. Tollefsen and M. K. Blank, unpublished observation.

quired to activate both the new inhibitor and AT III appeared to be two- to threefold greater than those in Fig. 5, a and b.

Studies in which purified AT III was substituted for plasma (Fig. 5, c and d) indicated (*a*) that the extent of formation of the M_r-85,000 complex was maximal at 0.01 U/ml of heparin and did not decrease with higher heparin concentrations, and (*b*) that the M_r-96,000 complex did not form even at the highest concentrations of heparin. Additional experiments using AT III-depleted plasma under identical incubation conditions (Fig. 5, e and f) demonstrated maximal formation of the M_r-96,000 complex at a heparin concentration of 1 U/ml; as in Fig. 4d, no band corresponding to the thrombin-AT III complex was seen.

Although not readily apparent from the autoradiograms in Fig. 5, a significant amount of radioactivity was present in the area of the gels between the M_r-85,000 and M_r-32,000 bands. As shown in Fig. 5 a, c, and e (open symbols) the radioactivity in this region paralleled that present in the M_r-85,000 complex but not in the M_r-96,000 complex, and it may thus represent dissociation of a fraction of the thrombin-AT III complexes (40–50%) during electrophoresis. This phenomenon has been described previously and may be connected with the presence of nucleophiles in the Laemmli running gel (26). Initial attempts to separate the two complexes by electrophoresis in neutral phosphate buffers have been unsuccessful.[3]

Estimation of the quantity of the new heparin-dependent inhibitor in plasma. By increasing the amount of ^{125}I-thrombin incubated with a fixed quantity of plasma in the presence of 5 U/ml heparin, we demonstrated saturation of both the M_r-85,000 and M_r-96,000 complexes in SDS gels. The amount of thrombin incorporated into each complex was calculated from the specific radioactivity of the ^{125}I-thrombin B-chain. Analysis of samples from 16 normal subjects revealed the following: 46±13 μg (mean±SD) of thrombin in the M_r-96,000 complex and 93±13 μg of thrombin in the M_r-85,000 complex/ml of plasma at saturation. The plasma concentrations of the new inhibitor and AT III were then estimated from these values, on the assumption of a 1:1 molar complex of thrombin and inhibitor in each case and molecular weights of 36,600 (13), 59,000 (see Methods), and 72,000 (see Discussion) for thrombin, AT III, and the new inhibitor, respectively. Thus, we calculated a concentration of 150±21 μg/ml for AT III and 90±26 μg/ml for the second heparin cofactor. These values, particularly that of AT III, should be considered minimum estimates of the true plasma concentrations, as the dissociated ^{125}I-thrombin was ignored in this calculation. Indeed, a 50% higher value for the concentration of AT III (225±32 μg/ml) was obtained by assaying the same plasma samples by radial immunodiffusion.

DISCUSSION

Our results indicate that human plasma contains a substantial quantity of an unidentified heparin-dependent inhibitor of thrombin. Like antithrombin III (23), the new inhibitor forms a complex with the thrombin B-chain that is stable in the presence of SDS and β-mercaptoethanol at 100°C. We have shown that thrombin treated with diisopropylfluorophosphate (16) or hirudin (27) to block the proteolytic active site of the molecule fails to form a complex with the new inhibitor in plasma.[3] These experiments are consistent with the formation of a covalent bond between the protease and the inhibitor and suggest that the active site of thrombin is required to form the complex (cf. 23 and 28).

The new inhibitor differs from AT III in the following respects: (*a*) It combines with thrombin to form a complex of M_r 96,000 in reduced SDS polyacrylamide gel electrophoresis (Fig. 1); this complex migrates more slowly than the thrombin-AT III complex (M_r 85,000). (*b*) The M_r-96,000 complex does not form when thrombin is allowed to react with purified AT III in the presence or absence of heparin. (*c*) Antibodies directed against human AT III do not react with the M_r-96,000 complex (Fig. 2). (*d*) Finally, the M_r-96,000 complex is formed when thrombin is incubated with plasma immunologically depleted of AT III (Figs. 4 and 5).

Additional experiments established that the new inhibitor is not identical to any of the other known plasma protease inhibitors. Thus, the M_r-96,000 complex forms in approximately normal amounts in plasma samples immunologically depleted of α2-antiplasmin, α2-macroglobulin, $\overline{C1}$ inactivator, α1-antichymotrypsin, or inter-α-trypsin inhibitor (Fig. 4). Although we were unable to remove α1-antitrypsin from plasma by this technique, we have shown that the M_r-96,000 complex does not react with antibodies directed against α1-antitrypsin (Fig. 2) and that the complex between thrombin and α1-antitrypsin migrates slightly more rapidly in SDS gels than the thrombin-AT III complex.[3] In addition, the M_r-96,000 complex was found in the plasma of two patients with combined Factor V/VIII deficiency,[3] which indicates that the complex is not due to the inhibitor of protein C recently described by Marlar and Griffin (29). It is also clear that the new inhibitor is not identical to "protease-nexin," the heparin-dependent thrombin-binding protein of fibroblasts described by Baker et al. (30), since the apparent molecular weights of the two complexes are quite different (96,000 vs. 68,000).

In the experiments illustrated in Fig. 5, we have allowed the reaction of a limiting amount of thrombin with plasma to go to completion in the presence of varying concentrations of heparin. The simplest interpretation of the results is that at higher heparin concentrations thrombin reacts more rapidly with the new inhibitor than with AT III. Heterogeneous heparin preparations derived from porcine intestinal mucosa contain many molecules that are inactive toward AT III (3). Whether the same subfractions of heparin activate both AT III and the new inhibitor remains to be determined. The observation that the new inhibitor is fully activated at 1 U/ml heparin in AT III-depleted plasma compared with 20 U/ml in unabsorbed plasma (Fig. 5, a and e) suggests that the two antithrombins might be competing for the same biologically active heparin species. Alternatively, as Jordan et al. (3) have pointed out, the rate at which AT III reacts with thrombin actually decreases at high heparin concentrations. This might allow the reaction with the new inhibitor to predominate.

At present, it is reasonable to assume that at levels of heparin achieved therapeutically by continuous intravenous infusion (i.e., 0.1–1.0 U/ml) (1), AT III is the major activated antithrombin in the plasma. The results shown in Fig. 5 support this hypothesis. It is unknown whether heparin secreted from mast cells or cell-surface mucopolysaccharides activate the new inhibitor. Thus, the two heparin-dependent antithrombins may be activated under different circumstances in vivo.

Previous investigators have separated plasma proteins by ion-exchange chromatography or electrophoresis and have claimed to resolve heparin cofactor activity from AT III (7–9). Although in general agreement with these reports, our studies differ in two important respects. We added thrombin directly to plasma and then separated the resulting complexes under denaturing conditions, thus minimizing the likelihood of artifacts such as proteolysis that might occur during the fractionation of plasma. Furthermore, we characterized the complexes immunologically.

Our results are at variance with more recent reports claiming that AT III accounts for virtually all of the heparin-dependent antithrombin activity of plasma. Rosenberg and Damus (23) reported that 92% of the heparin cofactor activity of heat-defibrinated plasma was removed by anti-AT III immunoglobulins. Preliminary experiments from our laboratory indicate that the new heparin-dependent inhibitor is substantially inactivated under the conditions of heat defibrination.[3] Holmer et al. (31) have also used antibodies to remove AT III from plasma. They found essentially no heparin cofactor activity in the AT III-depleted plasma when tested with 0.04 U/ml heparin. Our results indicate that higher heparin concentrations are required to activate the new inhibitor. Recently, Hoogendoorn et al. (32) have removed AT III from plasma by absorption to heparin-Sepharose and have found that the absorbed plasma still had a prolonged thrombin time and activated partial thromboplastin time in the presence of heparin. Unfortunately, no attempt was made to quantify the capacity of the absorbed plasma to inactivate thrombin.

We recently have purified the protein that reacts with thrombin to form the M_r-96,000 complex in plasma.[4] The purified inhibitor has an M_r of 72,000 in the Laemmli gel system and does not react with antibodies to any of the recognized plasma protease inhibitors.[2] It inhibits the proteolytic and amidolytic activity of thrombin by forming a 1:1 molar complex with the protease. The rate of complex formation increases >1,000-fold in the presence of heparin. Studies are in progress to elucidate the specificity of the new inhibitor with respect to various proteases and mucopolysaccharides. The physiological role of the new inhibitor remains to be defined.

ACKNOWLEDGMENT

This research was supported by grants HLBI 14147 (Specialized Center for Research in Thrombosis) and HL 16634 from the National Institutes of Health.

REFERENCES

1. O'Reilly, R. A. 1980. Anticoagulant, antithrombotic, and thrombolytic drugs. *In* The Pharmacological Basis of Therapeutics. A. G. Gilman, L. S. Goodman, and A. Gilman, editors. Macmillan Publishing Co., Inc., New York. 6th edition. 1347–1366.
2. Rosenberg, R. D. 1977. Biologic actions of heparin. *Semin. Hematol.* **14:** 427–439.
3. Jordan, R., D. Beeler, and R. Rosenberg. 1979. Fractionation of low molecular weight heparin species and their interaction with antithrombin. *J. Biol. Chem.* **254:** 2902–2913.
4. Laskowski, M., Jr., and I. Kato. 1980. Protein inhibitors of proteinases. *Annu. Rev. Biochem.* **49:** 593–626.
5. Rent, R., R. Myhrman, B. A. Fiedel, and H. Gewurz. 1976. Potentiation of C1-esterase inhibitor activity by heparin. *Clin. Exp. Immunol.* **23:** 264–271.
6. Pensky, J., L. R. Levy, and I. H. Lepow. 1961. Partial purification of a serum inhibitor of C1-esterase. *J. Biol. Chem.* **236:** 1674–1679.
7. Ganrot, P. O. 1969. Electrophoretic separation of two thrombin inhibitors in plasma and serum. *Scand. J. Clin. Lab. Invest.* **24:** 11–14.
8. Briginshaw, G. F., and J. N. Shanberge. 1974. Identification of two distinct heparin cofactors in human plasma: separation and partial purification. *Arch. Biochem. Biophys.* **161:** 683–690.

[4] D. M. Tollefsen, D. W. Majerus, and M. K. Blank. Manuscript in preparation.

9. Briginshaw, G. F., and J. N. Shanberge. 1974. Identification of two distinct heparin cofactors in human plasma. II. Inhibition of thrombin and activated Factor X. *Thromb. Res.* **4:** 463–477.
10. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* **4:** 330–334.
11. Miletich, J. P., G. J. Broze, Jr., and P. W. Majerus. 1980. The synthesis of sulfated dextran beads for isolation of human plasma coagulation Factors II, IX, and X. *Anal. Biochem.* **105:** 304–310.
12. Owen, W. G., and C. M. Jackson. 1973. Activation of prothrombin with *Oxyuranus scutellatus scutellatus* (Taipan snake) venom. *Thromb. Res.* **3:** 705–714.
13. Fenton, J. W., II, M. J. Fasco, A. B. Stackrow, D. L. Aronson, A. M. Young, and J. S. Finlayson. 1977. Human thrombins: production, evaluation, and properties of α-thrombin. *J. Biol. Chem.* **252:** 3587–3598.
14. Thaler, E., and G. Schmer. 1975. A simple two-step isolation procedure for human and bovine antithrombin II/III (heparin cofactor): a comparison of two methods. *Br. J. Haematol.* **31:** 233–243.
15. Kurachi, K., G. Schmer, M. A. Hermodson, D. C. Teller, and E. W. Davie. 1976. Characterization of human, bovine, and horse antithrombin III. *Biochemistry.* **15:** 368–373.
16. Tollefsen, D. M., J. R. Feagler, and P. W. Majerus. 1974. The binding of thrombin to the surface of human platelets. *J. Biol. Chem.* **249:** 2646–2651.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:** 680–685.
18. Swanstrom, R., and P. R. Shank. 1978. X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes ^{32}P and ^{125}I. *Anal. Biochem.* **86:** 184–192.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
20. Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. Methods in Immunology. W. A. Benjamin, Inc., Reading, Mass. 3rd edition. 313–321.
21. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* **2:** 235–254.
22. Lau, H. K., and R. D. Rosenberg. 1980. The isolation and characterization of a specific antibody population directed against the thrombin-antithrombin complex. *J. Biol. Chem.* **255:** 5885–5893.
23. Rosenberg, R. D., and P. S. Damus. 1973. The purification and mechanism of action of human antithrombin-heparin cofactor. *J. Biol. Chem.* **248:** 6490–6505.
24. Fish, W. W., K. Orre, and I. Bjork. 1979. Routes of thrombin action in the production of proteolytically modified, secondary forms of antithrombin-thrombin complex. *Eur. J. Biochem.* **101:** 39–44.
25. Kessler, S. W. 1976. Cell membrane antigen isolation with the staphylococcal protein A-antibody adsorbent. *J. Immunol.* **117:** 1482–1490.
26. Jesty, J. 1979. Dissociation of complexes and their derivatives formed during inhibition of bovine thrombin and activated Factor X by antithrombin III. *J. Biol. Chem.* **254:** 1044–1049.
27. Bagdy, D., E. Barabas, L. Graf, T. E. Petersen, and S. Magnusson. 1976. Hirudin. *Methods Enzymol.* **45:** 669–678.
28. Owen, W. G. 1975. Evidence for the formation of an ester between thrombin and heparin cofactor. *Biochim. Biophys. Acta.* **405:** 380–387.
29. Marlar, R. A., and J. H. Griffin. 1980. Deficiency of protein C inhibitor in combined Factor V/VIII deficiency disease. *J. Clin. Invest.* **66:** 1186–1189.
30. Baker, J. B., D. A. Low, R. L. Simmer, and D. D. Cunningham. 1980. Protease-nexin: a cellular component that links thrombin and plasminogen activator and mediates their binding to cells. *Cell.* **21:** 37–45.
31. Holmer, E., G. Soderstrom, and L-O. Andersson. 1980. Properties of antithrombin III depleted plasma. I. Effect of heparin. *Thromb. Res.* **17:** 113–124.
32. Hoogendoorn, H., A. Cerskus, F. Ofosu, M. Blajchman, and J. Hirsh. 1980. Preparation and partial characterization of human plasma depleted of antithrombin III by heparin-Sepharose affinity chromatography. *Thromb. Res.* **20:** 77–83.