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Heparin Binding Domain of Antithrombin III: Characterization Using a Synthetic Peptide Directed Polyclonal Antibody†

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Received April 12, 1990; Revised Manuscript Received May 22, 1990

ABSTRACT: Antithrombin III (ATIII) is a plasma-borne serine protease inhibitor that apparently forms covalent complexes with thrombin. The interaction between ATIII and thrombin is enhanced several thousandfold by the glycosaminoglycan, heparin. We have previously proposed that the heparin binding site of ATIII resides within a region extending from amino acid residues 114-156 [Smith, J. W., & Knauer, D. J. (1987) *J. Biol. Chem. 262*, 11964-11972]. Computer-assisted analysis of this region revealed the presence of a 22 amino acid domain (residues 124-145), part of which shows a strong potential for the formation of an amphipathic helix: hydrophobic on one face and highly positively charged on the other. In the presence studies, polyclonal antisera were generated against a synthetic peptide corresponding to residues 124-145 in native human ATIII. Affinity-purified IgG from these antisera, as well as monovalent Fab's derived from them, specifically blocked the binding of heparin to ATIII. Additionally, occupancy of the heparin binding site by these same monovalent and bivalent IgG's at least partially substituted for heparin, accelerating linkage formation between ATIII and thrombin. These results provide the first immunological evidence that region 124-145 is directly involved in the binding of heparin to ATIII and that an antibody-induced conformational change within this region can mediate ATIII activation.

Antithrombin III (ATIII)[1] is a key regulatory molecule in the control of intravascular clotting (Rosenberg & Damus, 1973; Rosenberg, 1977). ATIII inactivates a number of serine proteases that participate in the cascade, but principally controls clot formation by acting as a suicide inhibitor of thrombin. Apparently, a covalent linkage is formed between ATIII and thrombin at the active-site serine of the protease, and this linkage formation is accelerated several thousandfold by heparin (Jordan et al., 1980). It should be noted, however, that this covalent linkage is only inferred from chemical stability, and has never been directly demonstrated. Evidence suggests that the mechanism of this enhancement is attributable both to a conformational change induced in ATIII by heparin, which renders it more susceptible to proteolytic cleavage by thrombin, and to multiple binding sites on the linear heparin polymer that bring ATIII and thrombin into close proximity (Stone et al., 1982; Einarsson & Andersson, 1977; Nesheim et al., 1986).

The structure of the heparin binding site of ATIII has been of considerable interest, since the elucidation of it structure is the first step in understanding the mechanism of heparin activation. Nuclear magnetic resonance studies (Gettins & Wooten, 1987), as well as analysis of CNBr fragments of ATIII (Rosenfeld & Danishefsky, 1986), suggest that the heparin binding site of ATIII resides within the first one-third to half of the amino-terminal end of the molecule. In support of this, chemical modification experiments have implicated that lysine residues 107, 125, 133, and 136 are involved in the binding of heparin (Liu & Chang, 1987; Peterson et al., 1987; Chang, 1989).

† This work was supported by Grant 2R01-GM34001 from the National Institutes of Health and Grant 86-S107 from the American Heart Association.
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[1] Abbreviations: ATIII, antithrombin III; Th, thrombin; ^{125}I-F-heparin, ^{125}I-labeled fluoresceinamine heparin; RP-HPLC, reverse-phase high-performance liquid chromatography; IgG, immunoglobulin G; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; SPDP, succinimidyl (2-pyridyldithio)propionate; DTT, dithiothreitol; ACN, acetonitrile; BSA, bovine serum albumin; NP40, Nonidet P-40; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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In previous studies, we employed a direct biochemical approach to identify and characterize a heparin binding site in ATIII. A radioiodinated derivative of heparin, ^{125}I-F-heparin (Smith & Knauer, 1987a,b), was used to visualize heparin binding peptides generated by protease V8 digestion of native ATIII (Smith & Knauer, 1987a,b). Separation of the V8-generated heparin binding peptides of ATIII by RP-HPLC revealed three peptides. The smallest of these peptides was isolated and sequenced, and corresponded to residues 114–156 in native ATIII. Amino acid composition data of the larger peptides revealed that they were incomplete digestion products of ATIII and contained residues 114–156.

In the present report, we have extended these studies by generating a 22 amino acid synthetic peptide identical with residues 124–145 in native human ATIII. We have raised antisera against this peptide and purified the IgG from the antisera by affinity chromatography on peptide$^{124-145}$–Sepharose. We have demonstrated that the purified IgG, as well as monovalent Fab's derived from it, specifically blocks the binding of heparin to ATIII. Additionally, these same IgG's and Fab's partially substitute for heparin, by activating ATIII. These results significantly extend our previous findings that residues 114–156 in native ATIII are involved in heparin binding by narrowing this region to residues 124–145, and by demonstrating that antibodies directed to this domain are capable of activating ATIII.

Materials and Methods

Materials. ATIII was purified from human plasma as previously described (Peterson & Blackburn, 1985). Thrombin was a generous gift from Dr. John W. Fenton. High-affinity heparin and low-affinity heparin were a gift from Dr. Michael Blackburn. SPDP, protein A, protein A–Sepharose, and immobilized papain were from Pierce Biochemicals. CNBr was from Aldrich Biochemicals. Ovalbumin and RIA-grade BSA were from Sigma. All chromatography resins and electrophoresis reagents were purchased from Bio-Rad. Na^{125}I was obtained from Amersham. All other common shelf reagents were from Calbiochem or Sigma. RP-HPLC was done on preparative and analytical-scale C-4 columns purchased from Western Analytical. Protein concentrations were determined spectrophotometrically at 280 nm using the following molar extinction coefficients: thrombin, 1.74 mL·mg^{-1}·cm^{-1} (Lundblad et al., 1976); human antithrombin, 0.65 mL·mg^{-1}·cm^{-1} (Peterson & Blackburn, 1985); rabbit IgG, 1.34 mL·mg^{-1}·cm^{-1} (Hurn & Chantler, 1980). Synthetic peptides were lyophilized to dryness after purification and weighed.

Peptide Synthesis and Purification. The peptides used in these studies were chemically synthesized on a Milli-Gen peptide synthesizer at Immunodynamics Corp., La Jolla, CA. The final product was purified by RP-HPLC on a C-4 column. The crude peptide mixture was loaded onto the column in 0.1% TFA in HPLC-grade water and eluted with a linear gradient of acetonitrile, 0–45%, over a 35-min period at a flow rate of 1 mL/min.

Preparation of Anti-Peptide Antisera. Prior to immunization, the peptide was haptenized to ovalbumin as a carrier to enhance the immune response. Ten milligrams of ovalbumin in 1 mL of PBS was reacted with three additions of a 10-fold molar excess of SPDP made as a 100× stock in absolute ethanol. By spectral analysis, we determined that each ovalbumin monomer contained approximately four SPDP groups (Pierce Biochemicals). A 10-fold molar excess of the synthetic peptide was reduced with DTT, and following removal of excess DTT, the peptide was added directly to the SPDP-derivatized ovalbumin and reacted overnight at 4 °C. Free peptide was not removed. The ovalbumin–peptide conjugate was mixed with an equal volume of Freund's complete adjuvant and emulsified. Each of three rabbits was injected with 2 mg of the conjugate. Three weeks later, the rabbits were boosted with unconjugated, purified peptide in Freund's incomplete adjuvant. Ten days after the boost, the antiserum was collected from each of the rabbits and assayed for peptide-specific antibodies as described below.

Immunoblot Assay. Antisera from the rabbits were tested for peptide-specific antibodies by dot blots on nitrocellulose (Burnett, 1981). Three micrograms of ATIII was blotted onto nitrocellulose as individual dots in a 3-μL volume. The nitrocellulose was blocked for 3 h at 37 °C in 3% BSA in PBS, pH 7.2. Strips of the nitrocellulose were then incubated with each of the preimmune or immune antisera overnight at 4 °C. The strips were then washed successively for 10 min with Tris–saline (10 mM Tris buffer/0.15% NaCl, pH 7.2), twice with Tris–saline containing 0.05% NP40, and finally with Tris–saline. Antigen-bound IgG's were detected by incubating the blots with 10^5 cpm/mL ^{125}I protein A for 1 h at room temperature. The unbound protein A was removed by washing as described above. The dried blots were exposed to X-ray film for 12 h at −70 °C in the presence of an enhancement screen. As a positive control, anti-ATIII polyclonal antisera were used to visualize immobilized ATIII.

Affinity Purification of Peptide-Specific IgG. The whole IgG fraction was purified on protein A–Sepharose as described previously (Howard & Knauer, 1987). Peptide-specific IgG was purified from the whole IgG fraction on a 5-mL affinity column of peptide immobilized to Affi-Gel 501 sulfhydryl resin. Briefly, this column was prepared by adding a 10-fold molar excess of reduced peptide directly to the washed resin, to allow a disulfide linkage to form between the resin and the peptide. Uncoupled peptide was removed by washing the column extensively with alternate changes of 0.1 M sodium acetate, pH 4.0, and 0.1 M sodium bicarbonate, pH 8.1 One hundred milligrams of the whole IgG fraction in PBS was loaded onto the column and recirculated overnight. The column was washed with PBS until the base line was reached and then eluted with glycine hydrochloride, pH 2.7. The IgG peak was collected, neutralized with Tris-HCl, pH 8.8, and dialyzed into PBS.

Fab Preparation. Monovalent Fab fragments were prepared by digestion of affinity-purified IgG with immobilized papain (Pierce Biochemicals), according to the manufacturer's specifications. Briefly, 1 mg of affinity-purified IgG was incubated for 4 h at 37 °C with 100 μL of papain immobilized to Sepharose. Following removal of papain beads by centrifugation, the solution was dialyzed into PBS and passed over protein A–Sepharose to remove Fc fragments and any remaining intact IgG. The Fab fragments were judged to be completely pure by SDS–PAGE.

Solid-Phase Heparin Binding Assay. Ten milligrams of ATIII was added to each well of a Nunc Immunoplate in a total volume of 50 μL of PBS. ATIII was allowed to adhere to the plate for 18 h at 4 °C. The plate was washed 4 times with PBS to remove unbound ATIII, and then blocked for 3 h at 37 °C with PBS containing 3 mg/mL RIA-grade BSA. The wells were preincubated for 45 min with the indicated concentrations of unlabeled heparin or antibody, and then an additional 30 min with ^{125}I-F-heparin. Unbound heparin was removed by four washes with PBS at 4 °C. Radioactivity was removed from the wells by solubilization with boiling 1 N NaOH, and quantitated by γ counting. A similar assay was used to compare the heparin binding affinities of peptides

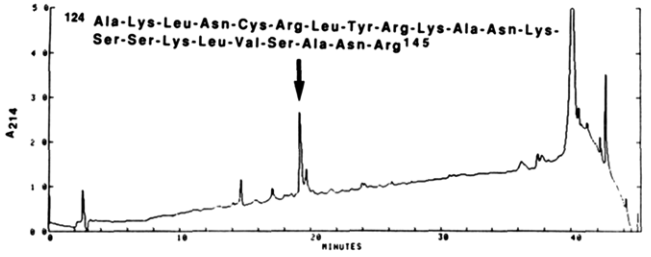

FIGURE 1: RP-HPLC purification of synthetic peptide$^{124-145}$. Ten micrograms of a crude preparation of synthetic peptide$^{124-145}$ was applied to an analytical-scale C-4 column in 0.1% TFA. The adsorbed material was eluted with a 0–45% linear ACN gradient in 0.1% TFA, at a flow rate of 1 mL/min. The A_{214} absorbance peaks indicated by the arrow were collected separately and subjected to sequence analysis. The larger of the two peaks has the amino acid sequence shown in the inset, which is identical with residues 124–145 in human ATIII.

124–145 and 117–137. The binding assay was conducted as described above, with the exception that the peptides were adsorbed to nitrocellulose rather than a protein binding plate.

Thrombin/ATIII Linkage Assay. ATIII (68 ng) was preincubated with the indicated concentrations of heparin, or IgG's, in 50 μL of PBS containing 100 μg/mL BSA for 40 min at 37 °C. Thirty nanograms of ^{125}I-thrombin was then added, and the reactions were terminated 30 s later by the addition of SDS–PAGE sample buffer. The high molecular weight complexes between ATIII and ^{125}I-thrombin were separated by SDS–PAGE on 7.5% gels as previously described (Baker et al., 1980).

Digestion and Separation of CNBr Peptides of ATIII. Six milligrams of ATIII was digested with CNBr as described (Rosenfeld & Danishefsky, 1987). Following removal of the CNBr by lyophilization, the peptides were separated by gel filtration on a 1.5 × 90 cm column of Bio-Gel P-60 in 0.1 M formic acid. Fractions of 1% bed volume were collected, and 200-μL aliquots were dried, resuspended in PBS, and tested for heparin and antibody binding in protein binding plates as described above.

Protein Radioiodinations. The fluoresceinated derivative of heparin was prepared and radioiodinated as previously described (Smith & Knauer, 1987a,b). Specific activities of the final preparations ranged from 70 000 to 100 000 cpm/ng. ^{125}I protein A and ^{125}I-thrombin were prepared as previously described (Low et al., 1980; Howard & Knauer, 1986) and routinely had specific activities ranging from 120 000 to 150 000 cpm/ng and from 15 000 to 20 000 cpm/ng, respectively.

Results

Peptide Synthetic and Characterization. A synthetic peptide corresponding to amino acid residues 124–145 in native ATIII was chemically synthesized (Figure 1, sequence). The final peptide product was extracted from the crude mixture and analyzed by RP-HPLC. Shown in Figure 1 is the elution profile of 10 μg of the crude peptide mixture from a C-4 reverse-phase column developed with a linear ACN gradient from 0 to 45%. The A_{214} absorbance peaks, indicated by the arrow at approximately 19 min. were handed-collected and analyzed for amino acid sequence by automated Edman degradation. The larger of the two peaks, which accounts for 80% of the total peptide, is identical with the amino acid sequence 124–145 in native ATIII. The smaller peak, which represents 20% of total peptide, is a truncated form, arising because of a decreased coupling efficiency at lysine-125 (data not shown). Lysine-125 is the 21st of 22 amino acids coupled, since synthesis is initiated from the carboxy terminus. Despite

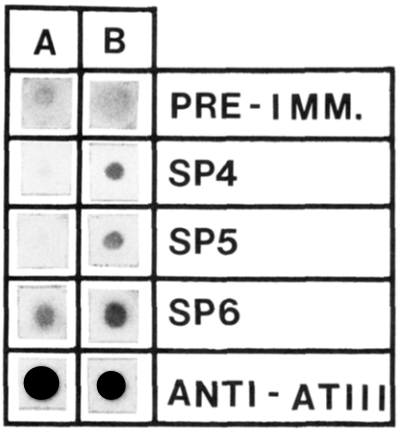

FIGURE 2: Immunoscreening of antisera raised against peptide$^{124-145}$. Three micrograms of ATIII was applied to replicate dots on nitrocellulose. The individual dots were incubated with each of the indicated antisera at a 1:100 dilution. Following an incubation with ^{125}I protein A to detect bound IgG's, the nitrocellulose dots were dried and exposed to X-ray film to produce the autoradiogram shown. The ATIII dotted in row A was boiled in a 0.05% SDS solution containing DTT prior to application to the nitrocellulose. Shown for comparison as a positive control is ATIII incubated with anti-ATIII polyclonal antisera at 1:100 dilution.

the similarity of this truncated form to complete 124–145 sequence, it was removed by preparative-scale HPLC on a C-4 column using the same parameters shown in Figure 1.

Characterization of Antisera against Peptide$^{124-145}$. In order to generate antisera, peptide$^{124-125}$ was haptenized to ovalbumin (see Materials and Methods) and injected into three rabbits. The initial coupling and primary injection were with a peptide mixture containing both the full-length and truncated forms of the peptide, shown in Figure 1. The rabbits were boosted with purified full-length peptide, and the antisera were collected and assayed for ATIII recognition by immunoblot analysis on nitrocellulose. All three of the rabbit antisera, SP4, SP5, and SP6, showed reasonable ATIII recognition at a 1:100 dilution (Figure 2). A pooled mixture of the preimmune sera from these rabbits gave no signal at the same dilution. Shown for comparison is the recognition of ATII by an anti-ATIII polyclonal antiserum at the same 1:100 dilution. Interestingly, if the ATIII was boiled in 0.05% SDS, prior to dotting to nitrocellulose (panel A, Figure 2), most of the antigenic recognition of the anti-peptide antisera was lost. In contrast, boiling in SDS actually increased the antigenic recognition of ATIII by the polyclonal antisera raised against human ATIII. These data suggest that the anti-peptide antisera recognize an exposed domain in native ATIII and that this domain is exposed when ATIII is immobilized to nitrocellulose under nondenaturing conditions.

To determine if the antisera raised against peptide$^{124-145}$ were specific to several sites within the peptide, or localized to a specific region, several N-terminal deletion peptides were tested for reactivity with the antibody. All of these peptides displayed strong cross-reactivity (data not shown). In contrast, when the antisera were tested for cross-reactivity with a peptide missing the last eight carboxy-terminal amino acids, peptide$^{117-137}$, immunoreactivity was completely lost. We were unable to detect binding, even at a 1:8 dilution of the antibody (Figure 3A). These data indicate that the antibody specifically requires the last eight residues within peptide$^{124-145}$ for binding. To eliminate the possibility that the loss of antibody recognition was due to a gross alteration in the conformation of peptide$^{117-137}$, or the inability of the peptide to adhere to nitrocellulose, we compared the binding of heparin to both peptides immobilized on nitrocellulose. When equal amounts

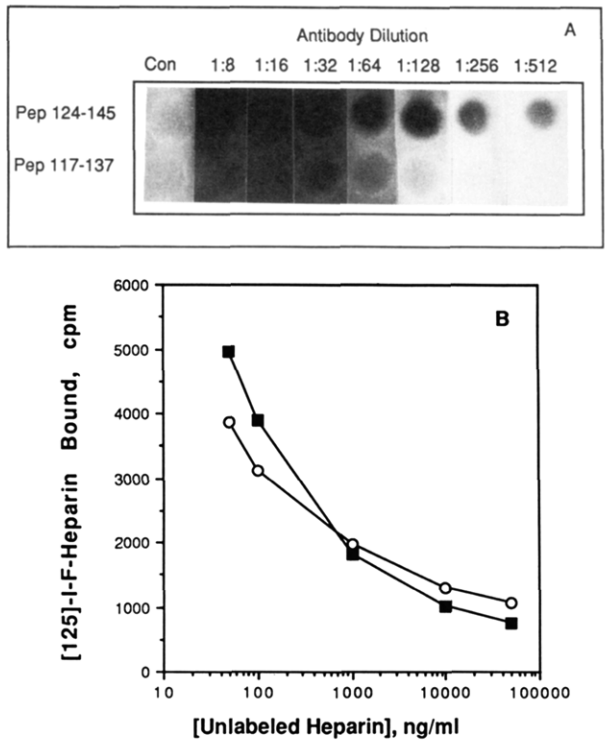

FIGURE 3: Specificity of anti-peptide$^{124-145}$ antibody. (Panel A) Three micrograms of peptide$^{124-145}$ and peptide$^{117-137}$ was dotted to nitrocellulose strips as described in Figure 2. The dots were incubated with anti-peptide$^{124-145}$ antisera over the indicated concentration range, and then washed. Bound IgG's were detected with ^{125}I protein A, followed by autoradiography. (Panel B) Three-microgram dots of peptide$^{124-145}$ (■) and peptide$^{117-137}$ (○) were prepared as described above. The individual dots were incubated with 10 ng/mL ^{125}I-F-heparin, and the indicated concentrations of unlabeled heparin for 1 h at 37 °C. Bound heparin was quantitated by γ counting the dots after excision from the nitrocellulose strips. Each point represents the average of duplicate samples. Individual samples did not vary more than 10% from the means. Maximum binding for both peptides, obtained in the absence of unlabeled heparin, was equal to the level of binding at the 50 ng/mL competition point. The first detectable competition was seen with 100 ng/mL unlabeled heparin.

of peptide$^{124-145}$ and peptide$^{117-137}$ were immobilized to nitrocellulose, both bound significant and nearly equal amounts of ^{125}I-F-heparin (Figure 3B). These data demonstrate that the loss of antibody recognition was not due to differences in the amounts of the peptides adhered to nitrocellulose. In both cases, the binding of ^{125}I-F-heparin was 80% specific, and competed for in a similar manner by increasing concentrations of unlabeled heparin, suggesting the same order and approximate binding affinity. Thus, it is unlikely that these two peptides have a highly altered conformation on nitrocellulose. Taken together, these data suggest that the lack of antibody recognition of peptide$^{117-137}$ can most likely be attributed to the direct interaction of the antisera with the last eight residues of peptide$^{124-145}$, which are absent in peptide$^{117-137}$.

Fractionation of Anti-Peptide$^{124-145}$ Antisera. Anti-peptide$^{124-145}$ was fractionated on a peptide affinity column to separate the immune and nonimmune IgG fractions (see Materials and Methods). Since peptide$^{124-145}$ contains several lysine residues, and has an isoelectric point of 10.5, we were concerned that the affinity column might have ion-exchange properties. To demonstrate that the IgG fractionation described was due to antigenic recognition, the control experiment shown in Figure 4 was done. Three micrograms of ATIII and peptide was blotted onto nitrocellulose. After being blocked, each was incubated with preimmune IgG, column flow-through IgG (IgG not retained on the peptide affinity

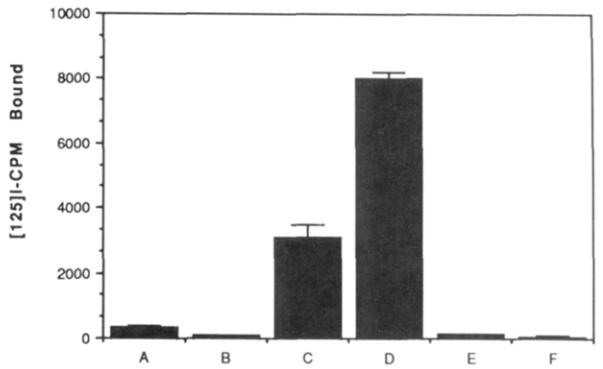

FIGURE 4: Fractionation of anti-peptide$^{124-145}$ IgG. Three micrograms of ATIII (A, C, and E) and peptide$^{124-145}$ (B, D, and F) was applied to nitrocellulose in replicate dots as described in Figure 2. The dots were incubated with 10 μg/mL preimmune IgG (A and B), 10 μg of affinity-purified IgG (C and D), or 10 μg of flow-through IgG (E and F) (see Materials and Methods for IgG fractionation procedure). Following a 30-min incubation with ^{125}I protein A, the dots were excised from the nitrocellulose and quantitated by γ counting. Each bar represents the average of triplicate samples. Standard deviations are indicated by the error bars.

column), or affinity-purified IgG. The blots were incubated with ^{125}I protein A to detect bound IgG. The dots were excised and quantitated by γ counting. Neither peptide nor ATIII was recognized by preimmune IgG, consistent with the data shown in Figure 2. Both ATIII and the peptide were recognized by the affinity-purified IgG, with 8000 and 4000 cpm of ^{125}I protein A retained, respectively. These data also demonstrate that the removal of peptide-specific IgG by the peptide affinity column was efficent, since the IgG fraction not retained on the affinity column (flow-through) failed to give a measurable signal above preimmune IgG. The recognition of ATIII by the affinity-purified IgG demonstrates that the purified IgG fraction was specific for the peptide, and was not purified on the basis of ion exchange.

Anti-Peptide IgG Is Specific for Region 124–145 in Native ATIII. To demonstrate that the anti-peptide IgG binds exclusively to the site in ATIII that includes region 124–145, we tested the ability of the IgG to recognize peptides of ATIII generated by CNBr digestion. Figure 5 is the Bio-Gel P-60 elution profile of a CNBr digest of ATIII. Fractions before 60-mL elution volume contained incomplete digestion products and are not shown. Four major peaks and several minor absorbance peaks were seen in the A_{280} absorbance profile (Figure 5). This is in agreement with predicted peptides based on the positions of methionine residues in human ATIII (Rosenfeld & Danishefsky, 1987). Samples of each of the peaks were subjected to SDS–PAGE (data not shown). Peak I has a molecular mass of 30 kDa, and represents an incomplete digestion product. This peptide was not characterized further. Peaks II, III, and IV have molecular masses of 18, 10.2, and 8.2 kDa, respectively. Each of these peptides corresponds to predicted CNBr cleavage peptides of ATIII (Rosenfeld & Danishefsky, 1987). Two micrograms of each of the peptides was immobilized in a 96-well protein binding plate and assayed for the binding of ^{125}I-F-heparin and anti-peptide IgG. Peak II displayed a significant level of heparin binding, whereas peaks III and IV were at background levels (Figure 5, inset panel A). Importantly, peak II was also the only peptide that was recognized by the antibody (Figure 5, inset panel B). Peak II, with a molecular mass of 18 kDa, corresponds to amino acid residues 100–251 in native ATIII. It therefore contains the peptide sequence 114–156 we had previously identified as a heparin binding region in ATIII, and the synthetic peptide sequence 124–145 that was used to

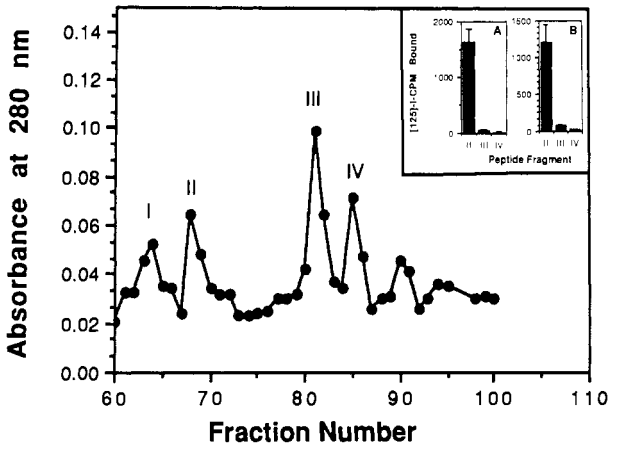

FIGURE 5: Separation of peptides generated by CNBr digestion of ATIII. Six milligrams of human ATIII was digested overnight with CNBr. The reaction mixture was lyophilized, resuspended in 1.0 mL of 0.1 M formic acid, and applied to a 1.5 × 90 cm Bio-Gel P-60 column. Fractions equal to 1% bed volume were collected and assayed for absorbance at 280 nm. Peptides eluting prior to fraction 60 represent large incomplete digestion products, and are not included in the profile. Peaks I–IV were subjected to SDS–PAGE on 15% gels and have been assigned the following molecular weights: I, 30K; II, 18K; III, 10.2K; IV, 8.2K. Inset: Peptides II–IV were resuspended in PBS, and 2 μg of each was used to coat the wells of a 96-well protein binding plate. Duplicate wells were incubated with 50 ng/mL ^{125}I-F-heparin (panel A) or anti-peptide$^{124-145}$ antibody at a 1:100 dilution (panel B) for 2 h at 37 °C. The wells incubated with ^{125}I-F-heparin were rinsed, solubilized with 1% SDS, and quantitated by γ counting. Nonspecific binding, determined by the addition of 200 μg/mL unlabeled heparin during the incubation in parallel wells, has been subtracted. Nonspecific binding was 20% of total. Wells incubated with antibody were rinsed and incubated with ^{125}I protein A (10^5 cpm) for an additional 30 min at room temperature. Following the removal of unbound protein A, the wells were rinsed, solubilized with 1% SDS, and quantitated by γ counting. Error bars represent 1 standard deviation from the mean.

generate the antisera. Together, peptides II, III, and IV comprise approximately 62% of the total mass of ATII. The other 38% of the mass is distributed among very small peptides, and we were unable to collect sufficient quantities of these peptides to assay. From these data, we conclude that within the 62% of ATIII assayed, the only binding of anti-peptide IgG is to a fragment that contains the peptide sequence used to raise the antisera. Although we cannot rule out the possibility that nonspecific interactions do take place between the anti-peptide IgG and AtIII in the remaining 38% of ATIII, these data clearly demonstrate that the anti-peptide IgG used in these studies binds primarily to a single specific site in ATIII.

Anti-Peptide IgG Blocks the High-Affinity Binding of Heparin to ATIII. Since the binding of anti-peptide IgG could be localized to a specific site in ATIII, we used this IgG to immunolocalize the heparin binding region in native ATIII. First, it was necessary to establish that our assay conditions measured the binding of heparin to a physiologically relevant site in ATIII. To make this assessment, we tested the ability of immobilized ATIII to discriminate between high-affinity heparin and low-affinity heparin in a competitive binding assay, using ^{125}I-F-heparin as the labeled ligand. The high- and low-affinity heparin preparations were made by repeated affinity purification of the high-affinity species from a single batch of heparin on ATIII–Sepharose. The binding of radiolabeled heparin to immobilized ATIII in this assay was efficiently competed by high-affinity heparin, and was reduced by greater than 50% at a concentration of 10 ng/mL (Figure 6A). In contrast, a 100-fold higher concentration of low-affinity heparin was required to achieve the same level of

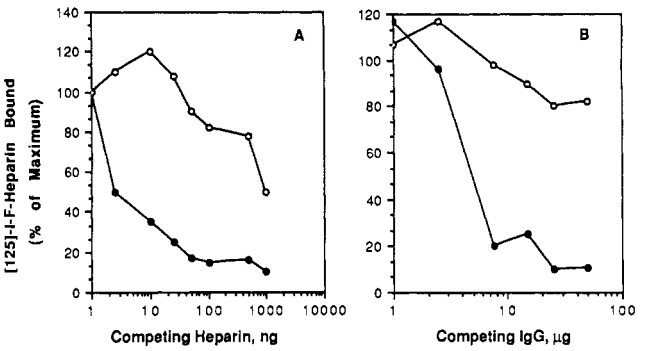

FIGURE 6: Blockage of ^{125}I-F-heparin binding by anti-peptide$^{124-145}$ IgG. (Panel A) Ten micrograms of ATIII in 50 μL of PBS was used to coat the wells of a 96-well protein binding plate. Following blocking, the wells were incubated for 30 min at 37 °C with the indicated amounts of high-affinity (●) or low-affinity (○) heparin. ^{125}I-F-Heparin (200 ng/mL) was then added, and the incubations were continued for an additional 30 min. Following the removal of unbound heparin, the wells were rinsed, solubilized with 1% SDS, and quantitated by γ counting. The dots have been normalized to control wells, which received only ^{125}I-F-heparin. (Panel B) The conditions were identical with those described in panel A, except that the preincubation was done in the presence of the indicated amounts of preimmune IgG (○) or anti-peptide$^{124-145}$ IgG (●) instead of unlabeled heparin. The data were normalized as described in panel A. Maximum binding in panels A and B, in the absence of competing ligand, was 12 000 cpm. Samples were done in duplicate, and did not vary more than 15% from the mean.

competition. The sharp increase in competition occurring between the 500 and 1000 ng/mL concentration of low-affinity heparin is probably due to a small amount of residual high-affinity heparin in the low-affinity heparin preparation. At the 1000 ng/mL point, a 1% contamination of high-affinity heparin in the low-affinity preparation would be expected to cause the same level of competition seen at the 10 ng/mL point with high-affinity heparin. These competition data are of particular importance, since they demonstrate that the solid-phase binding assay measures physiologically relevant heparin binding. If the binding of the radiolabeled heparin probe was simply due to electrostatic interactions, then we would not expect the differential competition curves for high- and low-affinity heparin.

The ability of affinity-purified anti-peptide IgG to compete for heparin binding was evaluated (Figure 6B) by using the same assay. Various concentrations of affinity-purified IgG and control IgG were preincubated with the immobilized ATIII for 30 min. ^{125}I-F-Heparin (10 ng/mL) was added, and the incubation was continued for an additional 30 min. Flow-through IgG, characterized in Figure 4, was unable to compete for the binding of heparin to ATIII. In contrast, affinity-purified IgG was a potent, concentration-dependent inhibitor of radiolabeled heparin binding (Figure 6B). Competition for heparin binding was first observed at 1 μg of IgG, and was complete at 7.5 μg. Since whole IgG is large in comparison to ATIII and is bivalent, we also tested Fab fragments derived from the affinity-purified IgG to compete for heparin binding (Figure 8A). As expected, the Fab fragments were as effective in competing for heparin binding as the native IgG. These data show that antibody bivalency is not required for competition, and also reduce the possibility that nonspecific steric hindrance plays a role in this competition, since the Fab fragment (40 kDa) is substantially smaller than native IgG (150 kDa). Taken together, the epitope mapping data strongly support our previous hypothesis that region 114–156 is directly involved in the binding of heparin to ATIII, and significantly narrow this region to residues 124–145.

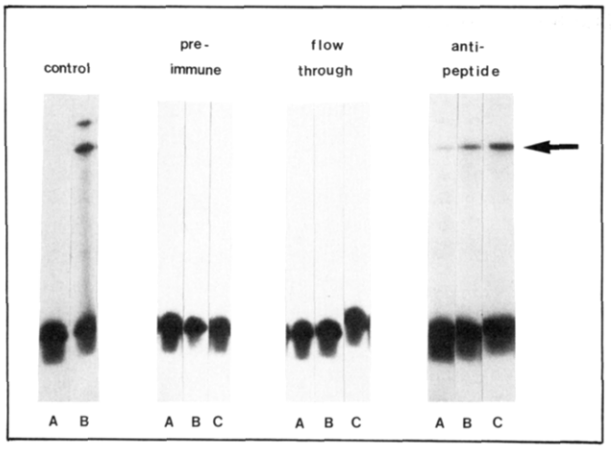

FIGURE 7: Anti-peptide$^{124-145}$ IgG accelerates linkage formation between ATIII and thrombin. ATIII (68 ng) was incubated with each of the IgG's in the indicated amounts (A, 1.5 μg; B, 7.5 μg; C, 15 μg) for 40 min at 37 °C. The total reaction volumes were 50 μL in PBS containing 100 μg/mL RIA-grade BSA. At the end of the 40-min incubation, 30 ng of ^{125}I-thrombin was added, and the reactions were allowed to proceed for 30 s. The reactions were stopped by the addition of 2× electrophoresis sample buffer and were analyzed by SDS–PAGE on 7.5% gels. The autoradiogram shown is an overnight exposure in the presence of an enhancement screen. The arrow indicates the position of ^{125}I-ThATIII complexes. The radioactivity near the gel front is uncomplexed ^{125}I-thrombin. As a control, complex formation was analyzed in parallel reactions where the preincubation was done in the absence (control lane A) or presence (control lane B) of an equimolar concentration of heparin.

Anti-Peptide IgG Accelerates Complete Formation between ATIII and Thrombin. Experiments were initiated to demonstrate that anti-peptide IgG could block the acceleration of complex formation between ATIII and thrombin by heparin. After several failed attempts, we considered the possibility that the anti-peptide IgG may be partially ligand-mimicking, i.e., may substitute for heparin and accelerate linkage formation. To test this hypothesis, an ATIII linkage assay was performed. ATIII was preincubated alone, or with heparin or 40 min at 37 °C. ^{125}I-Thrombin was added, and the incubation was continued for an additional 30 s. The reaction was terminated by the addition of SDS–PAGE sample buffer. In parallel reactions, ATIII was preincubated with various concentrations of preimmune, affinity-purified, or flow-through IgG (the depleted fraction obtained during the affinity purification of peptide-specific IgG). Similarly, ^{125}I-thrombin was added, and reactions were terminated 30 s later by the addition of SDS–PAGE sample buffer. The reactions were analyzed for complex formation between ATIII and ^{125}I-thrombin by SDS–PAGE on 7.5% gels (Figure 7). In the control lane A, there were no visible complexes formed in the absence of heparin during the 30-s incubation. In the presence of heparin, however, a significant amount of high molecular weight ^{125}I-Th/ATII complexes was formed (control lane B). At the three concentrations of IgG's tested, 1.5, 7.5, and 15 μg, there was no enhancement of complex formation by preimmune or flow-through IgG. In contrast, there was a marked enhancement of complex formation in the presence of anti-peptide IgG. Regions corresponding to ^{125}I-Th/ATII complexes were excised and quantitated by γ counting. There was a nearly linear increase in the acceleration of complex formation with increasing IgG concentration (data not shown). We have tested the IgG for cross-reactivity with thrombin, and found none (data not shown). This point is important, since a bivalent antibody that could simultaneously bind ATIII and thrombin might explain the acceleration data. Even though we could not detect cross-reactivity, the same experiment was repeated with Fab fragments to rule out any role of antibody bivalency (Figure 8B). As expected from the results of the blocking studies above, the Fab fragments were as efficient in accelerating linkage formation between ATIII and thrombin as bivalent IgG. We have attempted to characterize the acceleration kinetics, but have been unable to do so. Even though the IgG used in these studies is directed toward a small peptide, and is therefore probably against a single epitope, there are several different affinity class IgG's directed against this epitope, making kinetic studies difficult. Regardless, these data clearly demonstrate that occupancy of this eight amino acid stretch in native ATIII is sufficient to mediate acceleration of the reaction between ATIII and thrombin.

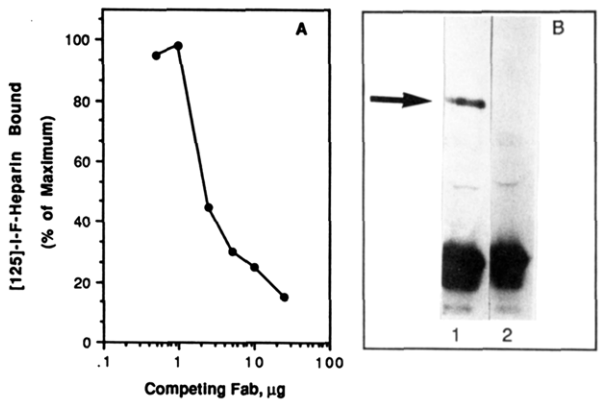

FIGURE 8: Monovalent Fab's derived from anti-peptide$^{124-145}$ IgG block the binding of heparin to ATIII and accelerate linkage formation. (Panel A) The ability of Fab fragments of anti-peptide$^{124-145}$ IgG to block the binding of ^{125}I-F-heparin to ATIII was assessed by using conditions identical with those described in the legend to Figure 7. The data have been normalized to control wells which received only ^{125}I-F-heparin, and are reported as percent of control. (Panel B) The acceleration activity of anti-peptide$^{124-145}$ Fab's on ^{125}I-ThATIII complex formation was tested by using conditions identical with those described in the legend to Figure 7. Lane 1, linkage formation in the presence of 5.0 μg of anti-peptide$^{124-145}$ Fab; lane 2, linkage formation in the absence of Fab.

Discussion

The present studies are an extension of our previous investigations into the identity and function of the heparin binding site in ATIII. We have previously shown that a heparin binding fragment of ATIII, isolated from a V-8 protease digest, included amino acids 114–156 in native ATIII. The major caveat to this approach is that we may have liberated a highly basic region of ATIII by proteolytic digestion, that was not in the proper conformation in native ATIII to bind heparin. To address this question and to further narrow the size of the site of heparin binding, we have mapped the heparin binding site in native ATIII using a synthetic peptide directed antibody.

The peptide chosen, representing residues 124–145, is a smaller region within the 114–156 fragment we originally identified and characterized by amino acid sequence analysis. This choice was based on chemical modification studies that suggested a role for lysine residues 125, 133, and 136 in heparin binding (Liu & Chang, 1987; Peterson et al., 1987; Chang, 1989). In the present studies, we have shown that affinity-purified IgG raised against peptide$^{124-125}$ specifically blocks the binding of heparin to ATIII. Since the binding assay we used allowed us to discriminate between high- and low-affinity heparin binding to ATIII, we concluded that the blocking we observed was due to the occupation of the physiologically relevant site in native ATIII by the antibody.

Additionally, Fab fragments derived from the affinity-purified IgG were as effective as the native IgG. These data rule out any requirement for IgG bivalency, and reduce the possibility of nonspecific steric hindrance playing an important role in the blocking reaction. It should be cautioned, however, that given the relative size of the Fab fragment to native ATIII, that steric hindrance cannot be absolutely ruled out. Our interpretation of the results are based on several supporting lines of evidence including the specificity of the antibody, the reactivity of the antibody with a specific eight amino acid sequence in ATIII, and the ability of the antibody to partially substitute for heparin.

Even though the IgG fraction used in these studies was made against a pure synthetic peptide and affinity-purified on the same peptide immobilized to Sepharose, we felt it necessary to demonstrate that the binding was specific to this region in ATIII. This was accomplished by purifying the three major CNBr digest peptides of ATIII, and demonstrating that both heparin binding and IgG binding were restricted to the 18-kDa peptide, residues 100–251, which included our peptide sequence, 124–145. These data are in agreement with those of Rosenfeld and Danishefsky, who demonstrated that this peptide is the only one of several generated by CNBr digestion that purifies on heparin–Sepharose (Rosenfeld & Danishefsky, 1987).

We also obtained an unexpected, but not unprecedented, result in the course of these studies. The same IgG that blocked heparin binding also partially mimicked the action of heparin on ATIII. Preincubation of this IgG with ATIII, followed by the addition of ^{125}I-thrombin, markedly enhanced the formation of high molecular weight ^{125}I-ThATIII complexes. This result supports the hypothesis that at least part of the accelerating effect of heparin on ATIII is mediated through an induced conformational change.

Previous studies have shown an increase in the fluorescence of Trp-49 upon heparin binding, indicating that a conformational change occurs in this region (Blackburn et al., 1984). In addition, naturally occurring ATIII variants, Toyoma (47, Arg to Cys) (Koide et al., 1984) and Basel (41 Pro to Leu) (Chang & Tran, 1986), suggest that this region of ATIII is involved in heparin binding. Using a computer-generated model of ATIII, based on the crystal structure of α_1-antitrypsin, it has been suggested that the major heparin binding domain of ATIII includes Arg-47 from helix A, helix D, which includes residues 124–132, and adjacent lysine residue 136 (Borg et al., 1988; Huber & Carrell, 1989). According to this model, these residues align to form a long stretch of positive charges which interact with the sulfate groups in the heparin polymer. In the case of factor Xa inhibition, occupation of this region by a heparin pentasaccharide induces a conformational change in ATIII sufficient to mediate activation. In the case of thrombin inhibition, however, longer heparin chains (12–15 units) are required. These longer heparin chains may directly influence the reactive site of ATIII by altering the local electrostatic environment.

The ability of the anti-peptide IgG used in these studies to accelerate the reaction between ATIII and thrombin may lie in the fact that it induces a conformational change in the random-coil region adjacent to helix D. This region includes lysine residues 133 and 136, and we have shown that the IgG used in these experiments requires residues 138–145 for binding. These residues lie to the carboxyl side of helix D, and project toward the reactive center of ATIII. Perhaps the anti-peptide IgG mimics heparin activation of ATIII by inducing a conformational change in these residues. This hypothesis is particularly attractive, since the ability of heparin to induce a conformational change in ATIII probably involves areas other than the highly ordered helix D region which would be expected to have minimal flexibility. This is also consistent with kinetic studies which demonstrate that the binding of heparin pentasaccharide to ATIII proceeds in two stages. The initial binding reaction is low affinity, followed by the establishment of a high-affinity state (Olson et al., 1981). It is possible that the ordered helix D accounts for the initial interaction and that the subsequent high-affinity state involves Arg-47, as well as positively charged residues extending beyond helix D.

Several models have been proposed to account for the acceleration of ATIII inhibitory activity. These include a thrombin activation model in which heparin binds to thrombin, making thrombin more accessible to ATIII inhibiton (Machovich, 1975; Griffith, 1979); an ATIII activation model, in which ATIII is conformationally altered upon heparin binding, and is thus more susceptible to proteolytic attack (Rosenberg & Damus, 1973; Einarsson & Andersson, 1977; Villanueva & Danishefsky, 1977); and a template model in which both thrombin and ATIII bind simultaneously to heparin as a requisite step in catalysis (Nesheim, 1983). Although there is good evidence for the template model, it should be noted that this model does not exclude conformational changes. The IgG activation data presented in this report demonstrate that at least part of the ATIII activation toward thrombin can be accounted for by a conformational change induced in ATIII. Since our antibody apparently recognizes eight residues (138–145), it will now be feasible to further examine ATIII activation using site-directed mutagenesis. In conjunction, we have recently begun to screen a bank of monoclonal antibodies made against peptide$^{124-145}$ for accelerating activity, so the kinetic aspects of activation can be further explored by using a single affinity class IgG.

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Evidence That Arginine-129 and Arginine-145 Are Located within the Heparin Binding Site of Human Antithrombin III

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Received February 14, 1990; Revised Manuscript Received April 30, 1990

ABSTRACT: Arginyl residues of human antithrombin III have been implicated to involve in the heparin binding site [Jorgensen, A. M., Borders, C. L., & Fish, W. W. (1985) *Biochem. J. 231*, 59–63]. We have performed chemical modification of antithrombin with (*p*-hydroxyphenyl)glyoxal (HPG) in order to determine the locations of these arginine residues. Antithrombin was modified with 12 mM HPG in the absence and presence of heparin (2-fold by weight to antithrombin). In the absence of heparin, about 3–4 mol of arginines/mol of antithrombin were modified within 60 min, and the modification led to the loss of 95% of the inhibitor's heparin cofactor activity as well as heparin-induced fluorescence enhancement and 50% of its progressive inhibitory activity. In the presence of heparin, the extent of modification was diminished by 30% and modified antithrombin retained approximately 70% of its heparin cofactor activity. Peptide mapping and subsequent sequence analysis revealed that selective HPG modification occurred at Arg^{129} and Arg^{145} and that their modifications were protected upon binding of heparin to antithrombin. We conclude that Arg^{129} and Arg^{145} are situated within the heparin binding site of human antithrombin III.

Antithrombin III (AT-III)[1] is a plasma glycoprotein and is the most important protease inhibitor that regulates the blood coagulation cascade [for reviews, see Rosenberg (1977) and Bjork and Lindahl (1982)]. AT-III inhibits thrombin as well as factors IXa, Xa, and XIa, and its inhibitory activity is greatly enhanced in the presence of heparin, a negatively charged polysaccharide. The precise mechanism of the heparin function remains to be defined. It has however been well established that heparin must bind to AT-III in order to exert its enhancing effect as an anticoagulant (Rosenberg & Damus, 1973; Villanueva & Danishefsky, 1977) and that high-affinity heparin in general displays high anticoagulant activity. Binding of heparin to AT-III induces a conformational change of the inhibitor (Einarsson & Andersson, 1977; Olson et al., 1981) and leads to the exposure of Lys^{236} for chemical modification (Chang, 1989). The consequence of this heparin-induced conformational change is at this stage still debated. It has been proposed either to activate the reactive site of AT-III (Rosenberg & Damus, 1973) or to tighten the heparin–thrombin complex (Olson et al., 1981; Peterson & Blackburn, 1987).

The heparin binding site of AT-III is thought to consist of clusters of basic amino acids. Four lysyl residues, Lys^{107}, Lys^{114}, Lys^{125}, and Lys^{136}, were shown to directly participate in the heparin binding site of AT-III (Peterson et al., 1987; Liu & Chang, 1987a; Chang, 1989). In addition, arginyl residues have also been implicated. A single amino acid replacement at Arg^{47} (Arg to Cys, His or Ser) impairs the heparin binding ability of AT-III congenital variants (Koide et al., 1984; Duchange et al., 1987; Borg et al., 1987). These findings confirm that Arg^{47} is essential for the integrity of the heparin binding site but do not conclusively prove that Arg^{47} is involved in direct heparin binding. Jorgensen et al. (1985) showed that the complete loss of the heparin cofactor activity of AT-III was accompanied by the modification of approxi-

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[1] Abbreviations: AT-III, human antithrombin III; HPG, (*p*-hydroxyphenyl)glyoxal; S-DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanoazobenzene-2′-sulfonic acid; DABITC, 4-(*N*,*N*-dimethylamino)-4′-isothiocyanoazobenzene; DABTH, 4-(*N*,*N*-dimethylamino)-4′-thiohydantoylazobenzene; HPLC, high-performance liquid chromatography.

0006-2960/90/0429-8957$02.50/0