A Heparin Binding Site in Antithrombin III
IDENTIFICATION, PURIFICATION, AND AMINO ACID SEQUENCE*

Jeffrey W. Smith and Daniel J. Knauer
From the Department of Developmental and Cell Biology, University of California, Irvine, California 92717

A heparin-binding peptide within antithrombin III (ATIII) was identified by digestion of ATIII with Staphylococcus aureus V8 protease followed by purification on reverse-phase high pressure liquid chromatography using a C-4 column matrix. The column fractions were assayed for their ability to bind heparin by ligand blotting with 125I-F-HRH, as previously described (Smith, J. W., and Knauer, D. J. (1987) Anal. Biochem. 160, 105-114). This analysis identified at least three fractions with heparin binding ability of which the peptide eluting at 25.4 min gave the strongest signal. Amino acid sequence analysis of this peptide gave a partially split sequence which was consistent with regions encompassing amino acids 89-96 and 114-156. These amino acids are present in a 1:1 molar ratio which is consistent with a disulfide linked Cys-95-Cys-128. This peptide has the high positive charge density within the molecule. A hydropathy plot of ATIII was generated using a method similar to that of Kyte and Doolittle (Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132). This plot indicates that amino acid residues 126-140 are exposed to the exterior surface of the molecule. Based on these data, we suggest that the region corresponding to amino acid residues 114-156 is a likely site for the physiological heparin-binding domain of ATIII. We also conclude that the proposed disulfide bridges within the protein are suspect and should be re-examined (Peterson, T. E., Dudek-Wojciechowska, G., Sotrup-Jensen, L., and Magnussen, S. (1979) in The Physiological Inhibitors of Coagulation and Fibrinolysis (Collen, D., Wiman, B., and Verstraeten, M., eds) pp. 43-54, Elsevier Scientific Publishing Co., Amsterdam).

ATIII is a plasma-borne protease inhibitor which inactivates a number of serine proteases involved in the coagulation cascade (1-4). ATIII inactivates serine proteases by forming a covalent ester-linked complex through the active site serine of the protease (5). The inhibition of proteases by ATIII is relatively slow in the absence of heparin but is enhanced as much as 10,000-fold in its presence (6). This enhancement is presumably mediated by the binding of heparin to ATIII, which induces a conformational change within the inhibitor (7).

Several structural features of ATIII are necessary for the heparin-enhanced inhibition of proteases by ATIII. For example, selective reduction and alkylation of the disulfide bridge between Cys-239 and Cys-422 prevents the heparin-enhanced thrombin inhibitory activity but does not interfere with the ability of ATIII to inhibit thrombin in the absence of heparin (8). Other lines of evidence show that the region spanning amino acid residues 41-49 is essential for the heparin-enhanced activity of ATIII. Chemical modification of the tryptophan at position 49 disallows the interaction between ATIII and heparin (9). Additionally, natural mutations of ATIII have arisen which do not display heparin-enhanced inhibitory activity of serine proteases. ATIII Toyama and ATIII Basel have substitution mutations at positions 47 and 41, respectively, and neither of these mutants display characteristic heparin-enhanced thrombin inhibition (10, 11).

Other evidence indicates that denaturation of ATIII with 0.7 M guanidinium chloride reversibly blocks heparin-enhanced thrombin inactivation (12). Predictions of a helical regions within ATIII which may be disrupted by guanidinium chloride treatment, and which contain lysines that may be essential for heparin binding, point to amino acid residues 282-289 as the heparin-binding domain of ATIII.

We present direct and theoretical evidence that the region encompassing amino acid residues 114-156 is a likely region for the physiological heparin-binding domain within ATIII. This peptide was originally identified by Cleveland digestion of ATIII, followed by ligand blotting with 125I-F-HRH, as described (1). Subsequently, we have purified this peptide using reverse-phase HPLC and confirmed its ability to bind heparin by dot blotting with 125I-F-HRH. Amino acid sequence analysis of this region corresponds to amino acid residues 89-96 and 114-156 within ATIII. We propose that these regions are disulfide linked and that the reported disulfide bridges have been incorrectly assigned.

A computer-generated plot of the charge density along ATIII shows that the region including amino acid residues 123-141 has the highest positive charge density in the mole-

* This work was supported in part by Research Grant 1 R01-GM34001-03 from the National Institutes of Health, and Grant-in-Aid 88-5107 from the California Chapter of the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Partially supported by National Institutes of Health Training Grant T3 CA09054. Present address: Dept. of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

1 The abbreviations used are: ATIII, antithrombin III; 125I-F-HRH, 125I-fluoresceinamine highly reactive heparin; RP-HPLC, reverse-phase high pressure liquid chromatography; PBS, phosphate-buffered saline.
Heparin-Binding Site of ATIII

MATERIALS AND METHODS

Chondroitin sulfate from bovine trachea, Tween 20, fluoresceinamine and CNBr were purchased from Sigma. Trifluoracetic acid and acetic acid were obtained from Pierce Chemical Co. The C-4 column matrix (10-μm pore size) was purchased from Vydac. Bac-Gel-F-100 and Affi-Gel-heparin were purchased from Bio-Rad. Porcine mucosal heparin (sodium salt) was obtained from Behring Diagnostics. Staphylococcus aureus V8 protease was purchased from Miles. Protein-binding plates were purchased from Nunc. Nitrocellulose was from Schleicher & Schuell, and 125I-Na (IMS-30) was purchased from Amersham. lodogen was from Pierce Chemical Co. HPLC separations were performed on a 15-20% acrylamide gradient SDS-PAGE gels. Human α-thrombin was a generous gift of Dr. John Fenton, New York State Department of Health, Albany, NY.

Protein Isolation—Human α-thrombin and peptide fragments of ATIII were radioiodinated using lodogen as previously described (14). Protein Digestion and Ligand Blotting—ATIII at 1 mg/ml in PBS was digested with various concentrations of S. aureus V8 protease at 1 mg/ml in PBS for 24 h at room temperature. Digested ATIII was analyzed on a 15-20% acrylamide gradient SDS-PAGE. Digests were electrophoretically transferred to nitrocellulose by the method of Burnette (15). Nitrocellulose blots were incubated with 125I-F-HRH, as described above. Briefly, blots were washed once with 0.2 M NaCl, 0.01 M Tris HCl, pH 7.3, and then incubated with 10 ng/ml of 125I-F-HRH in the same buffer containing 1 mg/ml chondroitin sulfate and 0.05% Tween 20 for 30 min. Blots were washed for 10 min, three times, in the same buffer and then exposed to x-ray film for 2-24 h.

Peptide Purification—ATIII at a concentration of 2 mg/ml was digested with S. aureus V8 protease at 1 mg/ml for 24 h at room temperature. This mixture was evaporated under a stream of nitrogen and resuspended in 0.1% trifluoroacetic acid in HPLC-grade water. Protein Digestion and Ligand Blotting—ATIII at 1 mg/ml in PBS was digested with various concentrations of S. aureus V8 protease at 1 mg/ml in PBS for 24 h at room temperature. Digested ATIII was analyzed on a 15-20% acrylamide gradient SDS-PAGE. Digests were electrophoretically transferred to nitrocellulose by the method of Burnette (15). Nitrocellulose blots were incubated with 125I-F-HRH, as previously described (1). Briefly, blots were washed once with 0.2 M NaCl, 0.01 M Tris HCl, pH 7.3, and then incubated with 10 ng/ml of 125I-F-HRH in the same buffer containing 1 mg/ml chondroitin sulfate and 0.05% Tween 20 for 30 min. Blots were washed for 10 min, three times, in the same buffer and then exposed to x-ray film for 2-24 h.

Preparation and Purification of 125I-F-HRH—Heparin was modified with fluoresceinamine after activation with CNBr, as described by Glabe et al. (16). Fluoresceinamine-derivatized heparin was radioiodinated and purified on ATIII-Sepharose, as described (1). The specific activity of this treatment was typically 50,000 cpm/μg of heparin.

Computer-generated Plots of Hydropathy and Charge Density—A computer program was written which allowed us to store the entire amino acid sequence of ATIII on an IBM personal computer. This program also enabled us to calculate the charge density of ATIII along its length and generate a plot of this using a 9-residue sample length. The charge density was calculated by dividing the net charge of side chains within a strand by the length of the strand. The same program was used to generate hydropathy plots according to the method of Kyte et al. (2) with one modification. Instead of adding the hydropathy scores for each amino acid along a strand to generate the score for the strand, we summed the hydropathy values and multiplied this sum by the strand length. Points were plotted at the midpoint of each strand.

Competitive-binding Assay Using High Affinity and Low Affinity Heparin—To test the specificity of the interaction between 125I-F-HRH and ATIII, 2 μg of ATIII were spotted directly on dry nitrocellulose in a volume of 3 μl. Each dot was excised from the nitrocellulose sheet and transferred to a polypropylene test tube. The dot blots were incubated with 1 μl of 0.01 M Tris HCl, pH 7.3, 0.2 μM NaCl + 1 mg/ml RIA grade bovine serum albumin. High affinity and low affinity heparin were added in amounts varying from 5 ng to 5 μg (as uronate) to the tubes in a constant volume. The blots were incubated for 1 h at 37 °C. 5 ng (as uronate) of 125I-F-HRH were added to each tube for an additional 30 min. The blots were washed four times with 1 ml of Tris-saline, followed by air drying, and exposed to x-ray film for 6 h at −70 °C in the presence of Cronex enhancing screen.

The purified heparin-binding peptide was assayed in a similar manner. 1 mg of ATIII was admixed with 1 μl of V8 protease for 24 h at 37 °C. The heparin-binding peptide was purified using RP-HPLC on a C-4 matrix as described under "peptide purification." The peak corresponding to the heparin-binding peptide was lyophilized and resuspended in 100 μl of distilled water. This mixture was diluted 1:10 in phosphate-buffered saline + 1 mg/ml of RIA grade bovine serum albumin. 2 μl of this mixture was spotted on dry nitrocellulose and assayed using the competition assay described for native ATIII.

RESULTS

Demonstration of the Specificity of the Interaction between ATIII and 125I-F-HRH—We have previously shown that 125I-F-HRH interacts specifically with ATIII whether in solution or on nitrocellulose (1). As an added proof, we have confirmed this using two additional methods. We chose to test the biological activity of fluoresceinamine-heparin by assaying its ability to promote covalent linkage formation between ATIII and 125I-thrombin. ATIII and 125I-thrombin were mixed in a 1:1000-fold concentration range. The reaction was allowed to proceed for 40 s, at which time it was stopped by addition of 0.05% SDS and immediate boiling of the samples. Duplicate samples were analyzed on SDS-PAGE followed by autoradiography. The regions of the gel corresponding to ATIII and 125I-thrombin complexes were excised and counted in a gamma counter. Fig. 1 depicts the results of this experiment and shows that the biological activity of native and fluoresceinamine-heparin are indistinguishable.

We have also confirmed that 125I-F-HRH interacts with ATIII at the physiological heparin-binding site when ATIII is immobilized on nitrocellulose. Low affinity and high affinity heparin were purified using affinity chromatography on ATIII-Sepharose, as previously described (13). Each of these types of heparin were tested for their ability to inhibit the binding of 125I-F-HRH to ATIII on nitrocellulose. 2 μg of...
ATIII and 125I-thrombin counter. ATIII-thrombin complexes were excised and counted in a gamma counter. Heparin were assayed for their ability to promote linkage between ATIII and 125I-thrombin in a Microfuge tube at a concentration of 1 × 10^{-8} M. Heparin and fluoresceinamine-heparin were added to the reaction mixes to yield final concentrations varying from 9.3 × 10^{-9} M to 9.3 × 10^{-6} M as uronate. Each reaction was allowed to proceed for 1 h at 37 °C in a volume of 1 ml. 125I-F-HRH (5 ng as uronate) was added for an additional 30 min. The blots were rinsed three times, dried, and exposed to x-ray film. The concentrations of competing heparin are as follows (as uronate): A, 5 ng; B, 12.5 ng; C, 25 ng; D, 50 ng; E, 125 ng; F, 250 ng; G, 500 ng; H, 2500 ng. The top panel corresponds to low affinity heparin and the bottom to high affinity heparin.

ATIII was immobilized on nitrocellulose as described under “Materials and Methods.” The dot blots were incubated with serial dilutions of high affinity and low affinity heparin for 1 h at 37 °C in a volume of 1 ml. 125I-F-HRH (5 ng as uronate) was added to each dot blot in the presence of high affinity and low affinity heparin for an additional 30 min. The dot blots were washed and exposed to x-ray film. The results shown in Fig. 2 demonstrate that high affinity heparin blocks the binding of 125I-F-HRH to ATIII in a concentration-dependent manner. Low affinity heparin, even at concentrations 1000 times greater than the probe, did not compete. These results conclusively show that 125I-F-HRH is interacting with ATIII at the physiological heparin-binding site.

Demonstration that the Interaction between 125I-F-HRH and ATIII Peptides Is Specific—125I-F-HRH is structurally distinct from native heparin because of the addition of the hydrophobic fluoresceinamine group, followed by the addition of 125I. We have previously shown that 125I-F-HRH interacts with native ATIII specifically (1). However, we also sought to prove that 125I-F-HRH binds to V8 protease-generated peptides of ATIII specifically, rather than through nonspecific charge interactions. ATIII was digested with V8 protease as previously described (1), except that the digestion time was extended to 40 min. Following digestion and SDS-PAGE, the peptides were electrophoretically transferred to nitrocellulose. The nitrocellulose strips were incubated with increasing amounts of unlabeled heparin (1–500 μg/ml dry weight) or chondroitin sulfate (10 μg–1 ng/ml dry weight) in 10 ml of 0.15 M NaCl, 0.01 M Tris HCl, pH 7.3, + 0.05% Tween 20 in an effort to compete for the binding of 125I-F-HRH. At the end of a 30-min incubation period, 150 ng of 125I-F-HRH in 40 μl of buffer was added directly to the incubation mixture for another 30 min. Blots were rinsed three times, dried, and

![Graph showing ATIII vs. Uronate (M x 10^{-3})]

**Fig. 1.** Comparison of the bioactivity of fluoresceinamine-heparin and native heparin. Native heparin and fluoresceinamine-heparin were assayed for their ability to promote linkage between ATIII and 125I-thrombin (Th). ATIII and 125I-thrombin were mixed in a Microfuge tube at a concentration of 1 × 10^{-8} M. Heparin and fluoresceinamine-heparin were added to the reaction mixes to yield final concentrations varying from 9.3 × 10^{-9} M to 9.3 × 10^{-6} M as uronate. Each reaction was allowed to proceed for 40 s, at which time the reaction was stopped by addition of 0.05% SDS in Laemmli buffer and boiling for 3 min. Duplicate samples were analyzed by autoradiography after SDS-PAGE. The regions of the gel corresponding to native heparin and fluoresceinamine-heparin.

**Fig. 2.** Competitive binding assay using 125I-F-HRH and high affinity and low affinity heparin. 2 μg of ATIII were immobilized on nitrocellulose dots and then incubated with serial dilutions of high affinity and low affinity heparin for 1 h. 125I-F-HRH (5 ng as uronate) was added for an additional 30 min. The blots were dried and exposed to x-ray film. The concentrations of competing heparin are as follows (as uronate): A, 5 ng; B, 12.5 ng; C, 25 ng; D, 50 ng; E, 125 ng; F, 250 ng; G, 500 ng; H, 2500 ng. The top panel corresponds to low affinity heparin and circles correspond to fluoresceinamine-heparin.

**Fig. 3.** Generation of heparin-binding peptides of ATIII by cleavage with S. aureus V8 protease. 10 μg of ATIII were incubated with the following amounts of V8 protease for 24 h at 22 °C: A, 0.02 μg; B, 0.04 μg; C, 0.1 μg; D, 0.2 μg; E, 0.4 μg; F, 1.0 μg; G, 2.0 μg; H, 10.0 μg. The incubation mixtures were separated on SDS-PAGE and electrophoretically transferred to nitrocellulose. Blots were incubated with 10 ng/ml of 125I-F-HRH as described and were exposed to x-ray film.
exposed to x-ray film for 3 h. This method of digestion and processing allows the visualization of five heparin-binding peptides. Binding of $^{125}$I-F-HRH is competed for by as little as 10 μg/ml of unlabeled heparin (Fig. 4). In contrast, chondroitin sulfate at concentrations of 1 mg/ml had no effect on the interaction between $^{125}$I-F-HRH. Thus, $^{125}$I-F-HRH interacts with intact ATIII and V8 protease-generated peptides of ATIII specifically.

**Purification of Heparin-binding Peptides Using RP-HPLC**—Initially, we attempted to purify heparin-binding peptides generated through V8 cleavage by electroelution from SDS gels. This method proved to be impractical because the smaller peptides would not stain using conventional staining techniques. Thus, the digests were analyzed using reverse-phase HPLC on a C-4 column matrix. Analytical digests were performed by digesting 200 μg of ATIII with 200 μg of V8 protease as described under “Materials and Methods.” This sample was analyzed using HPLC and generated a highly reproducible chromatogram (Fig. 5A). The fractions from this chromatogram were analyzed for their ability to bind $^{125}$I-F-HRH by dot blotting on nitrocellulose. It should be emphasized that dot blots were done in the presence of 1 mg/ml chondroitin sulfate. This precaution should have eliminated artifactual signals produced by simple charge-charge interaction between the peptides and the $^{125}$I-F-HRH. These dot blots identified 3 peaks that retain the ability to bind heparin. However, the peak eluting at 25.4 min gives a much stronger signal than the others (Fig. 5B, fractions 64–65). This peak was chosen for initial amino acid sequence analysis.

**Purification of Heparin-binding Peptides on Affi-Gel Heparin**—Since the peptide that exhibited heparin binding ability was purified using reverse-phase HPLC, we sought to confirm its ability to bind heparin by purifying it on Affi-Gel-heparin.

**Fig. 4.** Specificity of the interaction between $^{125}$I-F-HRH and V8-generated peptides of ATIII. 10 μg of ATIII were digested with 10 μg of V8 protease according to the method of Cleveland et al. (28). Digestions were electrophoretically transferred to nitrocellulose, and the nitrocellulose strips were incubated with the following concentrations of native heparin: A, 0; B, 1 μg/ml; C, 50 μg/ml; D, 500 μg/ml. An identical digest was prepared, and the nitrocellulose was incubated with the following amounts of chondroitin sulfate: E, 10 μg/ml; F, 100 μg/ml; G, 500 μg/ml; H, 1 mg/ml. Following the initial incubation, 5 ng/ml of $^{125}$I-F-HRH was added to each nitrocellulose strip for 1 h. Blots were washed and exposed to x-ray film.

**Fig. 5.** A, analysis of *S. aureus* V8 protease-generated peptides of ATIII using RP-HPLC. 200 μg of ATIII were digested with 200 μg of V8 protease for 24 h, and the resulting peptides were analyzed on RP-HPLC as described under “Materials and Methods.” The elution profile was generated by continuous monitoring of absorbance at 214 nm. B, dot blot analysis of HPLC fractions using $^{125}$I-F-HRH. A portion of each fraction from HPLC analysis of the ATIII digest was spotted on nitrocellulose and analyzed using $^{125}$I-F-HRH as described under “Materials and Methods.” Fractions 41–80 are shown; no positive signals were detected in fractions 1–40. Positive signals found in fractions 81–100 were found to be of relatively high $M_t$ (45,000) by iodination of the peptide and analysis on SDS-PAGE.
Fig. 6. Purification of heparin-binding peptides on Affi-Gel-heparin. ATIII (100 μg) was digested with V8 protease (100 μg) for 24 h and then incubated with a 100-μl suspension of Affi-Gel heparin beads. This mixture was washed five times with 200 μl of ice-cold PBS and then eluted with 100 μl of 1.5 M NaCl. Each sample was blotted on dry nitrocellulose and incubated with 125I-F-HRH as described. A, flow through; B–F, washes 1–5; G, 1.5 M NaCl elution.

Initially we sought to purify the peptides on heparin-Sepharose and then analyze them using reverse-phase HPLC. The amount of ATIII necessary to accomplish this precluded it as a viable approach. However, it was possible to digest small amounts of ATIII with V8 protease and incubate the digest with heparin-Sepharose in a Microfuge tube. The digestions were performed to completion using the conditions established in Fig. 2. The peptides which had no affinity for heparin were washed off the gel by rinsing several times with PBS. The remaining peptides were eluted with 1.5 M NaCl, and the eluate was spotted on nitrocellulose and analyzed as before with 125I-F-HRH. Small amounts of the heparin-binding peptides appeared in each of the washes, but the strongest signal was found in the eluate (Fig. 6).

Amino Acid Sequence Analysis of the Heparin-binding Peptide—Initial dot blot analysis of the ATIII peptides separated by RP-HPLC revealed three peaks which retained the ability to bind 125I-F-HRH. The peptide eluting at 25.4 min gave the strongest positive signal on dot blots and appeared to be one of the best-resolved peptides from the column profile. This peptide was chosen for amino acid sequence analysis. Subsequently, we have radioiodinated one of the other heparin-binding peptides and analyzed it using SDS-PAGE. The M, of this peptide was 45,000; thus, further characterization was not pursued. In addition, we have obtained preliminary amino acid composition data for the third heparin binding peptide which indicates that it encompasses the sequence of the peak eluting at 25.4 min.

Amino acid sequence analysis of the peptide eluting at 25.4 min was performed and 18 residues were resolved. A split sequence was obtained for the first six amino acids, as shown in Table I and Fig. 9. The first six pairs of amino acids were present in a 1:1 stoichiometry (Table I). Given the presence of cysteines 95 and 128, these results are consistent with two disulfide-linked peptides. This result is in conflict with the reported disulfide linkages in ATIII (3). It is unlikely that there was disulfide interchange, since at no time during the digestion or purification were the peptides exposed to reducing agents. It is also unlikely that these two peptides would co-migrate in the absence of a covalent linkage. We intend to resolve this discrepancy by re-examining the disulfide bridges within ATIII.

It is puzzling that the digestion with V8 protease cleaved between an alanine at position 88 and a methionine at position 89; however, this cleavage was highly reproducible. An identical C-4 elution profile was obtained when three separate preparations of ATIII and two batches of V8 protease were used. The digestion was performed at very high concentrations of V8 protease: 1 mg/ml of protease in a 1:1 weight ratio with ATIII. It is possible that at such high concentrations of protease some of the cleavage site specificity is lost. Alternatively, a contaminating protease may be present. The amino acid sequence of the smaller peptide extends to amino acid 95, but the actual cleavage probably occurred after amino acid 97, which is an aspartic acid, since V8 protease is known to cleave after aspartate.

The entire amino acid sequence of the heparin-binding peptide could not be determined, but we propose that the sequenced peptide extends to the glutamic acid at position 156. This proposed end point is based on two lines of evidence. First, analysis of digests with SDS-PAGE and ligand blotting with 125I-F-HRH indicate that the M, of the peptide is between 3000 and 6000 (Fig. 3), and this is consistent with the proposed end point. Additionally, we have reduced and alkylated the purified heparin-binding peptide according to the method of Lane (19). The peptide was repurified using the same RP-HPLC procedure as above. One peptide was recovered that retained the ability to bind 125I-F-HRH on nitrocellulose. This peptide was subjected to amino acid analysis as described under “Materials and Methods”, and the data are shown in Table II. A computer search was performed using the program PROTEIN.COM for regions within ATIII that were compatible with the composition data. These data confirm that the end point of the peptide is residue 156.

Two other peptides from the RP-HPLC purification generated weak positive signals on dot blot analysis with 125I-F-HRH. One of the peptides had a M, of 45,000 and was not examined further. The other peptide was subjected to amino acid analysis as described under “Materials and Methods”, and the results are shown in Table IIIA. Again a computer search for compatible regions was performed using PROTEIN.COM, and the results are shown in Table IIIB. The computer search was performed through strands 60–70 residues in length, and the acceptable error was set at 3%. 23 compatible sequences were found (Table IIIB). It should be emphasized that PROTEIN.COM essentially provides the user with a window within the protein strand that is compatible with composition data. Although the start sites and end points may not be exact, the strand from residues 114–156 is encompassed by all of the compatible sequences. Similar results were obtained when the computer search was per-
The heparin-binding peptide was reduced and alkylated and rechromatographed on a C-4 column. The peptide which retained the ability to bind to $^{125}\text{I}$-F-HRH was subjected to amino acid analysis. The amount recovered is shown in pmol. These data were converted to frequency of occurrence. The actual frequency of each amino acid in the peptide 114-156 is also shown.

| Amino acid | Amount recovered | Frequency (% of total) | Actual frequency (% of total) |
|------------|-----------------|-----------------------|-----------------------------|
| Asx        | 609             | 11.01                 | 12.50                       |
| Glx        | 502             | 9.07                  | 10.46                       |
| Ser        | 855             | 15.46                 | 18.01                       |
| His        | 142             | 2.56                  | 2.98                        |
| Arg        | 368             | 6.65                  | 8.25                        |
| Thr        | 223             | 4.03                  | 4.65                        |
| Ala        | 421             | 7.61                  | 9.07                        |
| Tyr        | 112             | 2.02                  | 2.37                        |
| Val        | 280             | 5.00                  | 6.08                        |
| Met        | 72              | 1.30                  | 1.56                        |
| Cys        | 16              | 0.28                  | 0.34                        |
| Ile        | 242             | 4.37                  | 5.10                        |
| Leu        | 622             | 11.24                 | 13.01                       |
| Phe        | 454             | 8.21                  | 9.81                        |
| Lys        | 651             | 11.41                 | 13.50                       |

Fig. 7. Competitive binding assay using the purified heparin-binding peptide and high and low affinity heparin. A small portion of the purified heparin-binding peptide (approximately 100 ng) was spotted on dry nitrocellulose, followed by incubation with serial dilutions of high affinity and low affinity heparin for 1 h. 5 ng of $^{125}\text{I}$-F-HRH (as uronate) were added directly to each dot blot for another 30 min. The dots were exposed to x-ray film, and the resulting autoradiograph was scanned with a densitometer to quantitate binding. Competition for binding by low affinity heparin is shown in open circles and high affinity heparin in dark circles. Heparin concentration is expressed as uronate.

formed on longer strand lengths. We conclude that the peptide eluting at 25.4 min in Fig. 4 is a subset of the peptide whose composition is shown in Table IIIA. Based on the data in Table II, we also conclude that the disulfide linkage is not necessary for heparin binding and that the heparin-binding site resides within residues 114-156.

The Interaction $^{125}\text{I}$-F-HRH and the Purified Heparin-binding Peptide Mimics the Physiological Interaction—Previous to purification of the heparin-binding peptide on RP-HPLC, we demonstrated that chondroitin sulfate does not block the binding of $^{125}\text{I}$-F-HRH to the V8 protease-generated heparin-binding peptides from ATIII. Although this implied that the interaction between $^{125}\text{I}$-F-HRH and the peptides is highly specific, we sought to confirm this hypothesis using the purified heparin-binding peptide. The purified heparin-binding peptide was immobilized on nitrocellulose and evaluated in a binding assay using $^{125}\text{I}$-F-HRH and high affinity and low affinity heparin. The individual dot blots were exposed to x-ray film as described under "Materials and Methods." However, the resulting autoradiograph was faint, so the individual data were scanned using a densitometer in order to quantitate binding. The peaks corresponding to dot density were excised and weighed on an analytical balance, and these data were converted to a percentage of maximum binding. The results of this experiment are depicted in Fig. 7. Both high affinity and low affinity heparin block the binding of $^{125}\text{I}$-F-HRH to the purified peptide, but high affinity heparin is clearly a better competitor. The fact that both species of heparin block binding has two possible explanations. It is conceivable that both primary and tertiary structure of ATIII contribute to the conformation of the heparin-binding domain of the molecule. Thus, the specificity that ATIII displays for distinct species of heparin may be partially conferred by the actual binding site and partially defined by the tertiary structure of the protein surrounding the binding site. It is also likely that low affinity heparin contains small amounts of high affinity heparin that readily interact with ATIII. Since the amount of the peptide that was spotted on the nitrocellulose was much lower than the amount of native ATIII used in Fig. 1, small amounts of high affinity heparin that may have been present in the low affinity heparin preparation may have effectively competed for the binding of $^{125}\text{I}$-F-HRH to the peptide. In either case, this experiment demonstrates that $^{125}$ F-HRH interacts with high affinity heparin with more avidity than low affinity heparin.

Computer Generation of Linear Charge Density and Hydropathy Plots—Our results regarding the heparin-binding site on ATIII represent a site which had not previously been proposed, and we therefore used two types of computer-generated analysis to test the plausibility of our data. It has been shown that the interaction between ATIII and heparin is dependent upon at least 1 lysine residue within ATIII (20). It is also reasonable to propose that the interaction between ATIII and heparin is largely mediated by specific charge interactions. With this hypothesis in mind, we sought to determine the regions on ATIII with the highest positive linear charge density. A computer program was written for this purpose. Charge density was calculated by summing the charges of amino acid side chains within a strand at pH 7.0. This sum was divided by the length of the strand, and the value was plotted at the midpoint of the strand (Fig. 8). The region of highest positive charge density is found in strands encompassing amino acid residues 123-141. This result complements our sequence data for the heparin-binding peptide.

As an added confirmation we sought to predict the orientation of this region using hydropathy plots according to the method of Kyte et al. (2). In this analysis amino acids were scored using the sum of the strand values; the sum was divided by the strand length to generate the plot in Fig. 8. This modification was performed in order to keep the data consistent with the plot of charge density. This plot confirms that the region extending from amino acid residue 123 to amino acid residue 141 has a high probability for being exposed rather than buried in the interior of the protein.

**DISCUSSION**

The mechanism of heparin-catalyzed thrombin inhibition by ATIII has been well characterized but has yet to be completely defined at the molecular level. We have devised a method for identifying heparin-binding peptides within heparin-binding proteins and have applied this methodology to identifying a heparin-binding region within ATIII (1). Several salient features of the interaction between ATIII and heparin...
Heparin-Binding Site of ATIII

**TABLE III**

Amino acid composition of the second low molecular weight heparin-binding peptide

Part A, the second low molecular weight heparin-binding peptide was hydrolyzed in vacuo in 6 M HCl, 0.1% phenol for 24 h at 115°C. Amino acid compositions were determined based on reverse-phase separation of phenylthiocarbamoyl derivatives using procedures outlined by Waters Associates. The amount of each amino acid recovered in pmol is shown. These data were converted to frequency of occurrence. Part B, a computer search was performed using the program PROTEIN.COM to find regions within ATIII that were compatible with these data. Strands of 60–70 residues in length from ATIII were tested for their compatibility with these data. The acceptable error was set at 3%, and 23 compatible strands were found. All of these encompass residues 114–156.

| Amino acid | Amount recovered | Frequency (% of total) |
|------------|------------------|-----------------------|
| Asx        | 1505             | 12.6                  |
| Gix        | 790              | 6.6                   |
| Ser        | 1498             | 12.6                  |
| His        | 319              | 2.7                   |
| Arg        | 909              | 7.6                   |
| Thr        | 643              | 5.4                   |
| Ala        | 977              | 8.2                   |
| Tyr        | 229              | 1.9                   |
| Val        | 446              | 3.8                   |
| Met        | 212              | 1.8                   |
| Cys        | 309              | 2.6                   |
| Ile        | 861              | 7.2                   |
| Leu        | 1041             | 8.8                   |
| Phe        | 786              | 6.6                   |
| Lys        | 1355             | 11.4                  |

### B

| Number | Sequence contents | Start | Length |
|--------|-------------------|-------|--------|
| 09601  | KDPDTSKGDQ1H...ETYQDISELVYGAK----| 00107 | 00062  |
| 09602  | PDDTISGKTSQDHF...TYQDISELVYGAKLQ---- | 00096 | 00062  |
| 09603  | DITISGKTSQDHFF...VQDISELVYGAKLQ---- | 00096 | 00062  |
| 09604  | TISGKTSQDHFF...QDISELVYGAKLQ---- | 00100 | 00062  |
| 09605  | ISGKTSQDHFFPA...DYQDISELVYGAKLQPL---- | 00111 | 00063  |
| 09606  | PKPDTISGKTSQDI...ETYQDISELVYGAK---- | 00106 | 00063  |
| 09607  | KPDTSKGDQ1H...TYQDISELVYGAKLQ---- | 00107 | 00063  |
| 09608  | PDDTISGKTSQDHF...VQDISELVYGAKLQ---- | 00108 | 00063  |
| 09609  | DITISGKTSQ1HFF...QDISELVYGAKLQ---- | 00109 | 00063  |
| 09610  | TISGKTSQ1HFF...DYQDISELVYGAKLQPL---- | 00110 | 00063  |
| 09611  | SISTAFAMTLKGC...KANKSSKLYSNAP---- | 00082 | 00064  |
| 09612  | VPKPDTSKGDQ1H...ETYQDISELVYGAKLQ---- | 00105 | 00064  |
| 09613  | KPDTSKGDQ1H...DYQDISELVYGAKLQ---- | 00107 | 00064  |
| 09614  | PDDTISGKTSQDHF...QDISELVYGAKLQ---- | 00108 | 00064  |
| 09615  | DITISGKTSQ1HFF...DYQDISELVYGAKLQPL---- | 00110 | 00064  |
| 09617  | LSISTAFAMTLKGA...KANKSSKLYSANRP---- | 00082 | 00065  |
| 09618  | SISTAFAMTLKGC...ANSSKLYSNARNLP---- | 00082 | 00065  |
| 09619  | EVPKPDTSKGDQ1H...ETYQDISELVYGAK---- | 00104 | 00065  |
| 09620  | VPKPDTSKGDQ1H...TYQDISELVYGAKLQ---- | 00105 | 00065  |
| 09621  | KPDTSKGDQ1H...VQDISELVYGAKLQ---- | 00106 | 00065  |
| 09622  | KPDTSKGDQ1H...VQDISELVYGAKLQ---- | 00107 | 00065  |
| 09623  | PDDTISGKTSQDHF...DYQDISELVYGAKLQPL---- | 00108 | 00065  |

should be mentioned before proposing a heparin-binding site. These features are discussed below.

Heparin has been shown to catalytically enhance the linkage of thrombin and ATIII (6). This enhancement is presumably brought about by the binding of heparin to ATIII. The binding of heparin to ATIII induces a conformational change within ATIII, which can be measured as an increase in tryptophan fluorescence (7). It has been postulated that this conformational change induces ATIII to enter an active state that is able to complex with thrombin more readily. Interestingly, it has also been demonstrated that this conformational change stabilizes the interaction between heparin and ATIII 300-fold (21).

Several structural features of ATIII have been implicated in the interaction with heparin. By considering the features in the context of what is known about the kinetics of the interaction, a model for the heparin-ATIII interaction can be proposed. The heparin-binding site is most likely highly positive in charge and is probably also exposed to the exterior surface of the molecule. Since the interaction between heparin and ATIII is stabilized by a conformational change, at least two other functional domains may exist which would be requisite to the interaction with heparin: 1) a "hinge" region from which the conformational change is originated, and 2) a stabilizing domain, which may come in proximity with the heparin as a result of the conformational change. This region may interact directly with heparin and thus stabilize the binding through a tertiary interaction. Indeed, one might propose several other mechanisms based on published data; we have settled on this mechanism because of its simplicity and testability.

Previous investigations have identified several structural features within ATIII which are necessary for the interaction with heparin. However, it has yet to be conclusively determined which, if any of these regions, is the heparin-binding domain within ATIII or if these regions correspond to the...
Heparin-Binding Site of ATIII

FIG. 8. Computer-generated plots of hydropathy and charge density of ATIII. Each amino acid within ATIII was scored according to its hydropathy (2) or the charge of its side chain at neutral pH. A computer program was written for an IBM personal computer that generated running averages of each, using an amino acid sample length of 9. Regions above the midline in the hydropathy plot tend to be in the interior of the molecule, whereas those below the midline tend to be exposed to the aqueous environment (2).

FIG. 9. Amino acid sequence of the heparin-binding peptide. The actual amino acid sequence of the heparin-binding peptide was determined for 18 amino acids (underlined). The proposed end point, as determined from $M_r$ and amino acid composition, is on the carboxyl side of the glutamic acid at position 156 (dashed lines).

Hinge or "stabilizing" regions. Two lines of evidence strongly imply that the region within ATIII from amino acid 41–49 is involved in heparin binding. Blackburn et al. (9) have shown that chemical modification of the tryptophan residue at position 49 blocks the binding of ATIII to heparin-agarose. This site-specific modification also abolishes the heparin-catalyzed inhibition of thrombin by ATIII. Two naturally occurring structural mutants of ATIII with impaired heparin-catalyzed inhibition of thrombin by ATIII. Two naturally occurring structural mutants of ATIII and ATIII Toyama has a Pro-Leu substitution at position 41, and ATIII Basel has an Arg-Cys substitution at position 47 (10, 11). These data are conclusive evidence that the region from amino acid 41–49 is involved in the interaction with heparin, although this involvement may be indirect. Interestingly, preliminary results from our laboratory indicate that ATIII which has been modified with HNB by the method of Blackburn will not bind $^{125}$I-F-HRH on nitrocellulose. However, following treatment with SDS, and boiling, this modified ATIII regains a large portion of its heparin binding ability. These data suggest that the region from amino acids 41–49 is indirectly involved in the binding of heparin.

Other investigators have shown that reduced and alkylated ATIII loses its ability to bind to heparin-Sepharose (8). In a refinement of this study, Longas et al. (22) were able to show that selective reduction and alkylation of one disulfide bond within ATIII abolished heparin-catalyzed thrombin inhibition.

The binding of heparin to ATIII is also dependent on the degree of sulfation and the net negative charge of the mucopolysaccharide (23, 24). Thus, it is reasonable to propose that lysines with ATIII are involved in this interaction. Rosenberg and Damus (25) have shown that modification of lysines within ATIII abolishes its ability to bind heparin. Peccon and Blackburn (20) more selectively modified lysines by the addition of pyridoxal-5'-phosphate and were able to show that heparin binding is blocked by modification of 1–2 lysines within ATIII.

Peterson and Blackburn (26) identified ATIII variants from normal human plasma which had a higher affinity for heparin-agarose than normal ATIII. These variants had the same
amino acid composition as normal ATIII but had reduced amounts of hexosamine, neutral sugars, and sialic acid.

Finally, Villanueva and Allen (27) demonstrated a two-domain structure for ATIII using denaturation with guanidinium chloride. One structural transition occurs at 0.7 M guanidinium chloride and is reversible. During this transition the heparin binding ability of ATIII is lost. By combining these observations with Chou-Fasman models of ATIII, Villanueva (12) has proposed that the region within ATIII extending from amino acid 282-289 corresponds to the heparin-binding site of ATIII.

We present the first direct evidence that the peptide corresponding to amino acid residues 114-156, disulfide linked to residues 89-96, within ATIII is sufficient to bind heparin. Although this site has not been previously suggested as a heparin-binding domain, closer examination of the structural features associated with this region are consistent with characteristics of the ATIII-heparin interaction. This region contains several lysines, and the two authors cited above have demonstrated that lysines are necessary for the heparin-enhanced inhibitory activity of ATIII. Additionally, this region contains three asparagine-linked carbohydrate moieties, at least one of which has been implicated in heparin interactions (26).

We have taken advantage of the fact that ATIII interacts with a specific species of heparin to further define the interaction between the purified heparin-binding peptide and 125I-F-HRH. High affinity heparin blocks the binding of 125I-F-HRH to the peptide 3- to 5-fold more effectively than low affinity heparin. This result confirms that the peptide constitutes a portion of the heparin-binding domain of ATIII. Higher concentrations of low affinity heparin did block the binding of 125I-F-HRH to the peptide. This may have been the result of contaminating high affinity heparin in the low affinity heparin preparation. Alternatively, the selectivity of ATIII for a specific heparin species may be the result of a tertiary interaction between distant regions of ATIII and heparin. We have also reduced and alkylated the purified heparin-binding peptide in order to determine if the disulfide linkage is necessary for heparin binding. The reduced and alkylated peptide was rechromatographed using RP-HPLC on a C-4 matrix. The peptide peak which retained the ability to bind 125I-F-HRH was subjected to amino acid analysis. The amino acid composition of this peptide was consistent with the sequence from residues 114 to 156 (Table II and Fig. 9). Our data are consistent with the hypothesis that amino acid residues 114-156 constitute a major portion of the physiological heparin-binding domain of ATIII.

Finally, and very importantly, computer-generated plots of linear charge density and hydropathy provide strong evidence that the region of amino acid residues 114-156 is a likely candidate for a heparin-binding domain. This is consistent with the hypothesis that the interaction between ATIII and heparin is partially dependent on specific electrostatic interactions. In addition, hydropathy plots predict that this region is also exposed to the aqueous environment in native ATIII. The data are particularly relevant because they indicate we have not exposed a latent heparin-binding site by digestion with V8 protease but rather have identified a site which is exposed to the aqueous environment. We believe that these are reasonable characteristics for a physiological heparin-binding domain. In contrast, the region containing residues 41-49 does not have a particularly high charge density, nor do hydropathy plots predict that it is highly exposed. Computer models of the hydropathy and charge density of the 282-289 region indicate that it has a weakly positive charge density and is not as highly exposed as amino acid residues 126-140.

The region within ATIII comprising residues 114-156 appears to be disulfide-linked to residues 89-96 and is sufficient for heparin binding. This is a likely region for the physiological heparin-binding domain within ATIII. Confirmation of this hypothesis will await chemical cross-linking of radiolabeled heparin to ATIII followed by digestion of the protein and sequencing of the labeled peptide. Future work will also include a re-examination of the assigned disulfide bridges within ATIII.

Acknowledgments—We would like to thank Charles Glabe and Paul DeAngelis for insightful conversations and for help with HPLC. We would also like to thank Kevin Hackitt for his expertise in computer programming. Information regarding the computer program used in this work may be obtained from Jeffrey Smith at the University of California, Irvine. We would also like to thank Dr. Michael Blackburn for providing low affinity and high affinity heparin. Finally, we would like to express great appreciation to Wilson Burgess for his valuable suggestions and expertise in amino acid sequence analysis.

REFERENCES

1. Smith, J. W., and Knauer, D. J. (1987) Anal. Biochem. 160, 105-114
2. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132
3. Petersen, T. R., Dudek-Wojnowska, G., Sottrup-Jensen, L., and Magnusson, S. (1979) in The Physiological Inhibitors of Coagulation and Fibrinolysis (Cullen, D., Wiman, B., and Verstraete, M., eds) pp. 43-54, Flavir Scientific Publishing Co., Amsterdam
4. Rosenberg, R. D. (1977) Semin. Hematol. 14, 427-446
5. Owen, W. S., Peisch, G. D., Yoder, E., and Pool, B. L. (1976) Thromb. Haemostasis 35, 87-95
6. Jordon, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) J. Biol. Chem. 255, 1098-1109
7. Villanueva, G. B., and Danishefsky, I. (1977) Biochem. Biophys. Res. Commun. 74, 803-809
8. Ferguson, W. S., and Finlay, T. H. (1983) Arch. Biochem. Biophys. 221, 304-307
9. Blackburn, M. N., Smith, R. L., Carson, J., and Sibley, C. C. (1984) J. Biol. Chem. 259, 939-941
10. Kicic, T., Odani, S., Takahashi, K., Ona, T., and Sakuragawa, N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 293-295
11. Chang, J.-Y., and Tran, T. H. (1986) J. Biol. Chem. 261, 1174-1176
12. Villanueva, G. B. (1984) J. Biol. Chem. 259, 2531-2536
13. Nielsen, M., Blackburn, M. N., and Mann, K. G. (1985) J. Biol. Chem. 261, 3214-3221
14. Glenn, K. C., Carney, D. H., Fenton, J. W., 11, and Cunningham, D. D. (1980) J. Biol. Chem. 255, 6609-6615
15. Burnett, W. N. (1981) Anal. Biochem. 112, 398-403
16. Glabe, C. G., Harry, P. R., and Rosen, S. D. (1983) Anal. Biochem. 130, 297-294
17. Lukas, T. J., Ivergen, D., Schleicher, M., and Wattersen, D. M. (1984) Plant Phys. 7, 763-790
18. Smith, J. W., Hackett, K., and Knauer, D. J. (1987) Anal. Biochem., in press
19. Lane, L. C. (1978) Anal. Biochem. 86, 444-485
20. Pecen, T. M., and Blackburn, M. N. (1984) J. Biol. Chem. 259, 938-938
21. Olson, S. T., Srinivasan, K. R., Bjork, I., and Shore, J. D. (1981) J. Biol. Chem. 256, 1073-1107
22. Longas, M. O., Ferguson, W. S., and Finlay, T. H. (1980) J. Biol. Chem. 255, 343-344
23. Riesemfeld, J., Thumberg, L., Höök, M., and Lindahl, U. (1981) J. Biol. Chem. 256, 2389-2394
24. Hurst, R. L., and Poe, M. (1985) J. Clin. Invest. 72, 1042-10453
25. Rosenberg, R. D., and Dams, P. S. (1973) J. Biol. Chem. 249, 6490-6505
26. Petersen, C. B., and Blackburn, M. N. (1985) J. Biol. Chem. 260, 610-615
27. Villanueva, G. B., and Allen, N. (1985) J. Biol. Chem. 258, 11010-11013
28. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102-1106