Identification of the Antithrombin III Heparin Binding Site*

Eva Erdsal-Badju, Aiqin Lu, Yancheng Zuo, Veronique Picard, and Susan Clark Bock‡

From the Temple University School of Medicine, Department of Microbiology/Immunology and The Sol Sherry Thrombosis Research Center, Philadelphia, Pennsylvania 19140

The heparin binding site of the anticoagulant protein antithrombin III (ATIII) has been defined at high resolution by alanine scanning mutagenesis of 17 basic residues previously thought to interact with the cofactor based on chemical modification experiments, analysis of naturally occurring dysfunctional antithrombins, and proximity to helix D. The baculovirus expression system employed for this study produces antithrombin which is highly similar to plasma ATIII in its inhibition of thrombin and factor Xa and which resembles the naturally occurring β-ATIII isoform in its interactions with high affinity heparin and pentasaccharide (Erdsal-Badju, E., Lu, A., Peng, X., Picard, V., Zendehrouh, P., Turk, B., Björk, I., Olson, S. T., and Bock, S. C. (1995) Biochem. J. 310, 323–330). Relative heparin affinities of basic-to-Ala substitution mutants were determined by NaCl gradient elution from heparin columns. The data show that only a subset of the previously implicated basic residues are critical for binding to heparin. The key heparin binding residues, Lys-11, Arg-13, Arg-24, Arg-47, Lys-125, Arg-129, and Arg-145, line a 50-Å long channel on the surface of ATIII. Comparisons of binding residue positions in the structure of P14-inserted ATIII and models of native antithrombin, derived from the structures of native ovalbumin and native antichymotrypsin, suggest that heparin may activate antithrombin by breaking salt bridges that stabilize its native conformation. Specifically, heparin release of intramolecular helix D-sheet B salt bridges may facilitate s123AhDEF movement and generation of an activated species that is conformationally primed for reactive loop uptake by central β-sheet A and for inhibitory complex formation. In addition to providing a structural explanation for the conformational change observed upon heparin binding to antithrombin III, differences in the affinities of native, heparin-bound, complexed, and cleaved ATIII molecules for heparin can be explained based on the identified binding site and suggest why heparin functions catalytically and is released from antithrombin upon inhibitory complex formation.

Pharmaceutical heparin is ubiquitously employed in modern medicine. Preparations of this sulfated glycosaminoglycan are given to increase anticoagulant activity in patients who have, or who are at risk for, venous and arterial thrombosis. Heparin is also used in laboratory blood sampling and in the increasingly common procedures of angioplasty, extracorporeal circulation, and hemodialysis.

The anticoagulant properties of heparin derive mainly from its ability to activate the serpin antithrombin III (ATIII),1 a key inhibitor of coagulation pathway enzymes including thrombin and factor Xa. Although it has been the subject of much attention and speculation, the mechanism for heparin activation of ATIII has remained an open question due to inadequate and imprecise information about the heparin binding site. This communication reports experiments that provide a high resolution definition of the ATIII heparin binding site and proposes a mechanism for ATIII heparin cofactor activity that is based on the new data and on information from several recently solved serpin crystal structures.

ATIII is a member of the serpin gene family (1) and uses a serpin-type protease inhibitor mechanism (2) to inactivate thrombin, factor Xa, and other coagulation enzymes. Covalent inhibitor-envelope complexes form when the P1 residue of a substrate-like sequence in the protruding reactive loop of a serpin interacts with the active site of its target enzyme. Proteinase inhibitor-function depends critically on mobility of the reactive loop, and in particular on its ability to stably insert into central β-sheet A during complex formation with target enzymes. Serpins in complex with their target proteinases are inferred to have their reactive loops partially inserted into their A-sheets as strand 4A (s4A) (3–6). This partially inserted conformation is intermediate between those of native serpins, which have 5-stranded A-sheets (7, 8), and latent and cleaved serpins which have fully 6-stranded A-sheets (9–15). As first described by Stein and Chothia (14), the conformational change associated with reactive loop insertion and inhibition is due to movement of a well defined fragment of serpin structure, consisting of helix F and strands 1, 2, and 3 of sheet A on joints formed by helices D and E. This s123AhDEF fragment moves, as a unit, away from the remainder of the serpin to open a gap between strands 3A and 5A of the native structure. It is into this gap that the reactive loop/s4A polypeptide is inserted (to varying degrees) in P14-inserted antithrombin (12, 13) and inhibitory (3–6), latent (11–13), and cleaved (9, 10) serpins.

In the absence of heparin, ATIII inhibits its target enzymes at rates which are 2–3 logs lower than those observed for most other serpins in the absence of cofactors. ATIII inhibitory activity can be potentiated, however, by heparin-like molecules (15). The physiological source of cofactor activity is heparan sulfate proteoglycans on and under the endothelium, which serve to localize and concentrate antithrombin on the vessel wall where activated coagulation enzymes are generated (16, 17). Additionally, pharmaceutical heparin may be administered to boost the anticoagulant activity of circulating antithrombin.

* This work was supported by National Institutes of Health Grant R01-HL30712 and American Heart Association Established Investigator Award 88-0261. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address and to whom correspondence should be addressed: University of Utah, Depts. of Medicine and Bioengineering, UUHSC-Pulmonary Division, 50 North Medical Dr., Salt Lake City, UT 84132. Tel.: 801-585-6521; Fax: 801-585-3355; E-mail: Susan.Bock@mcc.utah.edu.

1 The abbreviations used are: ATIII, antithrombin III; CA, α-carbon; aCT, antichymotrypsin; PAI-1, plasminogen activator inhibitor-1; s4A, strand 4A; HcII, heparin cofactor II.
A well-characterized highly sulfated pentasaccharide sequence (18–20) is responsible for most of the binding energy between heparin and ATIII (21). This pentasaccharide promotes a conformational change in ATIII which is almost fully responsible for heparin potentiation of factor Xa inhibition (21), whereas heparin potentiation of thrombin inhibition requires pentasaccharide-containing molecules of at least 18 units in length to approximate ATIII and the enzyme (22). The anticoagulant pentasaccharide has 10 negatively charged sulfate and carboxylate groups. Structure/function studies with synthetic pentasaccharides have shown that six of the negatively charged groups are essential for heparin activation of ATIII and that two others play significant roles in the process (reviewed in Ref. 23). This is in general agreement with an investigation of salt effects on the binding constant between high affinity heparin and ATIII which showed that five to six charged groups directly mediate the interaction between antithrombin and its cofactor (24).

In contrast to the antithrombin-binding pentasaccharide sequence of heparin, the heparin binding site of antithrombin is not precisely defined. Based on genetic variant, chemical modification, glycosylation isoform, and structural data, a cluster of positively charged residues in the helix D region has been proposed to interact with essential, negatively charged sulfate and carboxylate groups on heparin and to form the heparin binding site of ATIII (1, 12, 13, 25). Specific interactions of ATIII and the pentasaccharide have also been proposed based on docking studies using cleaved antithrombin structures (26–28). However, the validity of the latter models may be questioned because cleaved ATIII has greatly reduced heparin affinity (29). Together, the various models of ATIII’s heparin binding site have proposed that 19 different basic residues on the surface of antithrombin are involved in interactions with heparin. This number is significantly in excess of the estimated five to eight critical ionic interactions derived from studies of salt effects on the binding constant for high affinity heparin and antithrombin (24) and pentasaccharide structure/activity data (23).

ATIII is a glycoprotein with four N-glycosylation sites, and its heparin binding properties are sensitive to (i) whether oligosaccharide addition occurs at asparagine 135 (Asn135) and (ii) characteristics of the N-linked oligosaccharides that are present on the molecule. The naturally occurring β-isofor-m of ATIII lacks carbohydrate on asparagine 135 (30) because its NXS type consensus sequence is inefficiently glycosylated (31). The β-isof orm binds heparin more tightly than does α-isof orm due to effects of the Asn135 oligosaccharide on the heparin-induced conformational change which accompanies formation of the high affinity heparin-ATIII complex (59). Affinity of antithrombin for heparin is also affected by characteristics of attached carbohydrates. With the exception of small differences in sialylation, complex oligosaccharides attached to the four N-glycosylation sites of plasma-derived ATIII are identical (33, 34), and the plasma α- and β-ATIII isoforms elute cleanly from immobilized heparin. This is not the case, however, for recombinant antithrombins produced in mammalian tissue culture cells. Antithrombins expressed in BHK and CHO cell lines are heterogeneously glycosylated due to nonuniformity in host cell post-translational modification processes. This kind of glycosylation heterogeneity, in addition to α-ATIII/β-ATIII glycosylation heterogeneity caused by the wild type Asn135 NXS consensus sequence (31), causes the expressed antithrombin molecules to display a broad range of affinities for heparin (32, 35, 36). Consequently, it is problematic to determine the heparin affinities of ATIII variants produced in these expression systems, and they are not optimal for in vitro mutagenesis studies of the ATIII heparin binding site (35).

We have avoided the glycosylation and heparin binding heterogeneity problems associated with production of antithrombin in cultured mammalian cells by developing a baculovirus system that expresses physically and functionally homogeneous ATIII in insect cells. α/β glycoform heterogeneity was abolished by alanine substitution of Asn135 to generate a cDNA encoding a β-like ATIII. The remaining N-glycosylation consensus sequences are uniformly modified in the lepidopteran host cells, and purified by hat3.N135A migrates as a single band on SDS-polyacrylamide gel electrophoresis and isoelectric focusing gels. Bv.hat3.N135A is highly similar to plasma ATIII in its inhibition of thrombin and factor Xa in both the absence and the presence of heparin and resembles the β-ATIII isoform in its binding to high affinity heparin and pentasaccharide (37). These properties support its use as a base molecule for in vitro mutagenesis studies on the structural basis of ATIII-heparin interactions. This communication presents our initial work on alanine scanning identification of basic residues in the heparin binding site.

MATERIALS AND METHODS

ATIII Expression System and Site-directed Mutagenesis—The base molecule for this study was bv.hat3.N135A (37). The N135A substitution eliminates binding heterogeneity associated with partial glycosylation of asparagine 135 (31) and directly produces a homogeneous β-ATIII–like parent molecule with high base-line affinity for heparin (37). Basic-to-Ala substitutions were introduced using a one-tube polymerase chain reaction mutagenesis method (38). Following sequence verification and subcloning, variants were expressed as described previously (37).

Heparin Affinity Chromatography of ATIII Variants—The relative heparin affinities of variants in the basic-to-Ala substitution series were determined by binding cleaved supernatants to Bio-Rad EconoPac heparin cartridges and eluting with a NaCl gradient. The standard gradient was 0.7–3.1 mM NaCl in 20 mM phosphate, pH 7.4, 0.1 mM EDTA. Low affinity variants a.R47A, a.K125A and a.R129A were eluted with 0.1–1.5 mM NaCl. The designation a indicates that these substitutions were expressed on a bv.hat3.N135A base. ATIII presence in fractions was determined using Western blots prepared from 10% polyacrylamide gels of reduced samples. To accurately compare elution positions of the different variants, NaCl concentrations of individual column fractions were determined by diluting aliquots 1:100 with distilled water and measuring conductivity versus a standard curve. Elution patterns for different preparations of a given variant grown under standard culture conditions (37) and harvested at 80–90% confluence were highly reproducible. Low affinity variants from at least two different recombinant viruses (plaque) of each variant were prepared and yielded similar results for a given substitution.

Thrombin Complex Formation Assay for Protease Inhibitor Function—Protease inhibitor function was checked by incubating 10–μl aliquots of variant-containing lysates with 0, 14, 35, or 175 pmol of human thrombin. After 10 min at 37°C, reactions were subjected to non-reducing SDS-polyacrylamide gel electrophoresis and Western blotting with sheep anti-human ATIII Ig (Binding Site, Birmingham, UK) as described previously (31).

Structural Analysis—The alignment of Huber and Carrell (1) was used to identify which residues of ovalbumin and antichymotrypsin correspond to heparin binding residues of ATIII and their neighbors. For the case of antichymotrypsin, the accuracy of the Huber and Carrell alignment was verified by confirming that equivalent residues superposed in overlaid cleaved antichymotrypsin (10) and cleaved (28) and latent (12, 13) ATIII structures. QUANTA software (Molecular Simulations) was used for structure visualization and analysis.

An almost complete composite structure for P14-inserted antithrombin was assembled from superpositioned non-latent molecules of the independently solved human antithrombin dimer structures (12, 13), which are missing electron density in different areas. For N-terminal segments where density is inadequate in both human structures, coordinates were extracted from the structure of cleaved bovine ATIII (28), which was overlaid on the P14-inserted molecules with matching for the case of antichymotrypsin, the accuracy of the Huber and Carrell alignment was verified by confirming that equivalent residues superposed in overlaid cleaved antichymotrypsin (10) and cleaved (28) and latent (12, 13) ATIII structures. QUANTA software (Molecular Simulations) was used for structure visualization and analysis.

An almost complete composite structure for P14-inserted antithrombin was assembled from superpositioned non-latent molecules of the independently solved human antithrombin dimer structures (12, 13), which are missing electron density in different areas. For N-terminal segments where density is inadequate in both human structures, coordinates were extracted from the structure of cleaved bovine ATIII (28), which was overlaid on the P14-inserted molecules with matching for the region. Thus, the composite P14-inserted molecule used in these studies and shown in Figs. 2 and 3 consists of coordinates for (i) bovine antithrombin (28) molecule A residues 5–21 and 37–46 (equivalent to
Human ATIII 4–20 and 36–45, (ii) pdb1ant (12) molecule I residues 21–29 and 46–137, and (iii) pdb1ath (13) molecule A residues 138–431. The indicated regions of pdb1ant and pdb1ath were selected to generate a composite molecule containing the C terminus of helix D (for which density is missing in pdb1ant) and the P14 region of the reactive loop (for which density is missing in pdb1ant). However, with regard to the analysis of relationships between Lys125 and Arg129 and negatively charged residues in sheet B, it is noted that although the composite molecule helix D and sheet B coordinates were copied from pdb1ant and pdb1ath, respectively, Lys125, Arg129, Glu414, and Asp278 of overlaid pdb1ant and pdb1ath molecules superimpose, and the Lys125–Glu414 and Arg129–Asp278 CA distances are highly similar in the independently solved structures (see Table I).

### RESULTS

#### Heparin Binding Site Identification

Recognizing that further advances in understanding of the antithrombin heparin cofactor activation mechanism would be aided by a more precise understanding of the ATIII heparin binding site, we undertook to define it at high resolution by alanine scanning mutagenesis of basic residues in the helix D region. Seventeen different arginine and lysine residues were singly converted to alanines and expressed on the background of human ATIII (41, 42) data, and the involvement of Lys11, Arg13, and Arg24 in heparin binding was predicted by patient variants with pentasaccharide for binding to ATIII (45). We also found Arg145 to be an important heparin binding residue, in agree-

#### TABLE I

<table>
<thead>
<tr>
<th>CA distances between heparin binding residues Lys125 and Arg129 and negatively charged residues in sheet B (Å)</th>
<th>Lys125, Glu134</th>
<th>Arg129, Asp278</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native ovalbumin, Ref. 8, pdb1ova</td>
<td>(Asp98–His376)</td>
<td>(Glu102–Thr427)</td>
</tr>
<tr>
<td>Native antichymotrypsin, Ref. 7, coordinates provided by D. Katz and D. Christianson</td>
<td>(His98–Pro376)</td>
<td>(Thr102–Asn247)</td>
</tr>
<tr>
<td>Cleaved antichymotrypsin, Ref. 10, pdb2ach</td>
<td>(His98–Pro376)</td>
<td>(Thr102–Asn247)</td>
</tr>
<tr>
<td>P14-inserted antithrombin III</td>
<td>(His98–Pro376)</td>
<td>(Thr102–Asn247)</td>
</tr>
</tbody>
</table>

Overall, the Ala scan showed that only a limited number of basic residues in the helix D region are critical for heparin binding. The importance of Arg47, Lys125, and Arg129 had been strongly suggested by previous genetic (39, 40) and chemical modification (41, 42) data, and the involvement of Lys11, Arg13, and Arg24 in heparin binding was predicted by patient variants with N-terminal polypeptide heparin binding defects (43, 44) and epitope mapping of a monoclonal antibody that competes with pentasaccharide for binding to ATIII (45). We also found Arg145 to be an important heparin binding residue, in agree-

![Fig. 1. ATIII variant interactions with heparin and thrombin.](http://www.jbc.org/)

A, heparin affinity column elution profiles of bv.hat3.N135A parent molecule and 17 basic-to-alanine variants. Relative affinities of ATIII variants for heparin were determined by immunoblotting Laemmli gels of fractions collected during NaCl gradient elution. The blots were scanned and plots show relative intensities of the ATIII band (x axis) in successive fractions as a function of NaCl concentration (y axis). For the R47A, K125A, and R29A variants which elute at low salt concentrations where misfolded and polymerized ATIII also elutes (37), plots indicate position of functionally active variant molecules; elution positions of active ATIII were identified by incubating gradient fractions with thrombin and scanning the thrombin-antithrombin complex band. B, formation of SDS-stable thrombin complexes by low affinity variants. AT, antithrombin; cAT, cleaved antithrombin; T-AT, thrombin-antithrombin complex.
ment with previous chemical modification evidence (42).

In addition to demonstrating that certain basic residues interact strongly with heparin, our data contribute to definition of the binding site by eliminating other basic residues as major contributors to heparin binding. In contrast to previous descriptions of the heparin binding site (1, 12, 13, 25–28), we found that Lys\(^{129}\), Arg\(^{132}\), Arg\(^{133}\), Lys\(^{135}\), and Lys\(^{275}\) contribute minimally to ATIII affinity for heparin. Thus, as a whole, our data establish that basic residues in close proximity to each other exhibit distinct interactions with heparin and imply that a geometrically precise configuration of positive charges is required for heparin binding to ATIII. The number of basic residues that were critical for heparin binding was in the range (5–8) that had been predicted by binding constant salt dependence (24) and pentasaccharide structure/activity data (23).

Fig. 2 shows the structure of P14-inserted ATIII (12, 13) and the locations of heparin binding basic residues identified in the Ala scan. The seven basic residues that are most critical for ATIII heparin binding (blue side chains) define a site that is centered over Arg\(^{47}\) of helix A, Lys\(^{125}\) and Arg\(^{129}\) of helix D, and Lys\(^{11}\) and Arg\(^{13}\) of the N-terminal polypeptide and that extends around the molecule to include Arg\(^{145}\) in sheet A and Arg\(^{24}\) of the N-terminal polypeptide. The solid rendering in Fig. 2A shows that heparin binding site residues line a channel on the surface of ATIII. This channel is actually even deeper than it appears to be in the figure because of the presence of large, biantennary, complex oligosaccharides on asparagines 96, 135, 155, and 192 (yellow spheres) of the predominant \(\alpha\)-ATIII isoform. The length of the heparin binding channel is approximately 50 Å, corresponding to an oligosaccharide of about 10 units to span from Arg\(^{24}\) to Arg\(^{145}\). Pentasaccharide binding residues likely include a subset of those that are necessary for binding to the immobilized heparin used in our chromatography assay.

An additional interesting feature of the heparin binding site is illustrated in Fig. 2B, which shows a CA backbone rendering of P14-inserted ATIII. The s123AhDEF fragment (which moves as a unit to accommodate reactive loop strand 4A insertion (14)) has been drawn in ribbon format, and it is evident that several key heparin binding site residues (Arg\(^{13}\), Lys\(^{125}\), and Arg\(^{129}\)) lie at the interface of s123AhDEF and the remainder of the molecule. The in the “Discussion,” we shall propose that heparin binding to these interface residues disrupts intramolecular salt bridges that stabilize the native conformation of antithrombin. Disruption of the stabilizing salt bridges then allows s123AhDEF movement and generation of an activated heparin-ATIII complex that is conformationally primed for reactive loop uptake by central \(\beta\)-sheet A upon encountering a target proteinase.

**DISCUSSION**

The configuration of heparin binding residues and their relationship to other antithrombin structural elements were examined in P14-inserted (12, 13), latent (12, 13), and cleaved (28) ATIII crystal structures, as well as in the structures of a native antichymotrypsin variant (7) and ovalbumin (8) for approximation of the native ATIII heparin binding site. As discussed below, this analysis indicates that progressive incorporation of the reactive loop into sheet A is associated with specific changes in the local environment of key heparin binding site residues and with overall changes in heparin binding site configuration. Analysis of these differences provides a structural rationale for why heparin binding to antithrombin causes a protein conformational change that is associated with increased proteinase inhibitor activity and for why heparin functions catalytically and is released from antithrombin upon inhibitory complex formation.

**Heparin Disruption of Salt Bridges Stabilizing the Native**

**Conformation of ATIII—Heparin cofactor activation of antithrombin III is associated with a protein conformational change (21). Similarly, serpin proteinase inhibition is dependent on a conformational change, which in this case is known to involve the reactive loop and \(\beta\)-sheet A of the inhibitor (2). We therefore reasoned that analysis and comparison of heparin binding site configurations in native antithrombin and the partially s4A-inserted, P14-inserted ATIII molecule might provide information about the mechanism of ATIII heparin cofactor activation. Unfortunately, however, a native ATIII structure is not directly available. Attempts by two different groups (12, 13) to crystallize native antithrombin independently yielded crystals of a dimer containing one P14-inserted molecule and one latent ATIII molecule. Therefore, we analyzed the
Currently available native serpin crystal structures (a native antichymotrypsin variant (7) and native ovalbumin (8)) to obtain information about the structure of native ATIII. These structures have intact, completely external reactive center loops and five-stranded A-sheets, and we used their backbones as models for the backbone of native antithrombin.

Fig. 3 compares native (purple backbones with blue reactive loops) and s4A-inserted (yellow backbones with red reactive loops) serpin structures to show how the local environments of heparin-binding residues Lys125 and Arg129 change as a result of the s123AhDEF shift that accompanies reactive loop insertion. Analyses based on comparisons of P14-inserted antithrombin with native ovalbumin and cleaved antichymotrypsin (aCT) with native aCT are based on the alignment of Huber and Carrell (1) and by reference to ovalbumin and aCT structures superpositioned on cleaved and latent ATIII structures. Equivalent residues of ATIII/ovalbumin/aCT are Lys125/Asp98/His98, Glu 414/His376/Pro376, Arg 129/Gln102/Thr102, and Asp278/Thr247/Asn247.

Identifying amino acids that correspond with heparin-binding residues Lys125 and Arg129 and their neighboring amino acids in the native serpin structures suggests that salt bridges may connect Lys125 with Glu414 and Arg129 with Asp278 in native antithrombin. Inspection of P14-inserted ATIII and cleaved aCT reveals CA distances for these pairs are 2–3 Å longer than the corresponding distances in native ovalbumin and native antichymotrypsin (see Table I). From above analysis were ovalbumin (pdb1ova, Ref. 8), native antichymotrypsin P3-P3’ variant (coordinates kindly provided by D. Katz and D. Christianson, Ref. 7), P14-inserted ATIII (see “Materials and Methods”), and cleaved antichymotrypsin (pdb2ach, Ref. 10). The N-terminal polyepitope of P14-inserted ATIII is white. Ovalbumin and aCT residues corresponding to Lys125-Glu414 and Arg129-Asp278 of P14-inserted ATIII and the corresponding residues of cleaved antichymotrypsin are 2–3 Å longer than distances between the same residues of native ovalbumin and native antichymotrypsin (see Table I). From this we infer that intramolecular salt bridges (hatched lines in diagrams) connect Lys125 with Glu414, and Arg129 with Asp278 in native antithrombin, but are disrupted when the reactive loop inserts as strand 4A. The dual roles of Lys125 and Arg129 in both the binding of heparin and the native salt bridge stabilization of s123AhDEF suggest the heparin cofactor activation mechanism presented in Fig. 4. Structures used for above analysis were ovalbumin (pdb1ova, Ref. 8), native antichymotrypsin P3-P3’ variant (coordinates kindly provided by D. Katz and D. Christianson, Ref. 7), P14-inserted ATIII (see “Materials and Methods”), and cleaved antichymotrypsin (pdb2ach, Ref. 10). The N-terminal polyepitope of P14-inserted ATIII is white. Ovalbumin and aCT residues corresponding to Lys125-Glu414 and Arg129-Asp278 of P14-inserted ATIII and the equivalent residues of cleaved antichymotrypsin are 2–3 Å longer than the corresponding distances in native ovalbumin and native antichymotrypsin. A third salt bridge linking heparin-binding residue Arg47 (helix A) with Glu113 (helix D) may also form in native ATIII; however, this is less certain since residues equivalent to Arg47 are not present in the structures of ovalbumin or native aCT.

The relationships illustrated in Fig. 3 and Table I suggest a mechanism for heparin cofactor activation (Fig. 4) in which heparin binding to Lys125 and Arg129 (and possibly Arg47) disrupts two (or possibly three) salt bridges stabilizing the native conformation and frees the s123AhDEF fragment to shift in a manner that will promote reactive loop insertion. Thus, by uncoupling salt bridges that fix the native conformation, heparin is proposed to catalyze the transformation of the relatively unreactive native ATIII molecule into an activated species that is conformationally primed for reactive loop insertion and inhibited complex formation.

With respect to proposed stabilization of native antithrombin by salt bridges, we note a similarity to stabilization of native PAI-1 by vitronectin. In this case, bound vitronectin fixes the s123AhDEF fragment of PAI-1 in a native position by cross-linking s1A and helix E of s123AhDEF to helix C in the large fragment of the serpin, thereby preventing s4A insertion and formation of latent PAI-1 (46).
Diagrams representing the interacting antithrombin and heparin structures, the blue molecule. Basic residues of the ATIII heparin binding site are represented by i.e. of most other serpins (47–49), plasminogen activator inhibitor-1 (PAI-1) (50), their progressive inhibition rates are higher than is antithrombin's.

Native salt bridges may also explain why basal rates of ATIII target proteinase inhibition are substantially lower than those of most other serpins (i.e. $<10^4 \text{ M}^{-1} \text{ s}^{-1}$ versus $>10^6 \text{ M}^{-1} \text{ s}^{-1}$). Slow “progressive” activity in the absence of heparin may be a consequence of the need to break native salt bridges prior to reactive loop incorporation. In this regard, it is notable that a K125M antithrombin mutant exhibited a 2–3-fold increase in its rate of progressive factor Xa inhibition (53).

It is also of interest to note that although heparin cofactor II (HcII) (47–49), plasminogen activator inhibitor-1 (PAI-1) (50), and glial-derived nexin (51) also have glycosaminoglycan binding sites including residues equivalent to Lys125 and Arg129, homology modeling does not predict multiple intramolecular salt bridge stabilization of their native conformations by cross-linking of helix D and the B-sheet, and with the exception of HcII (54), their progressive inhibition rates are higher than is antithrombin’s.

With regard to heparin cofactor II, it is noted that salt bridges involving helix D residues are believed to participate in glycosaminoglycan regulation of HcII thrombin inhibition, but by a different mechanism. In this case, dermatan sulfate potentiation of the inhibitory reaction has been proposed to involve disruption of salt bridges between positively charged D helix residues and an N-terminal hirudin-like sequence of HcII (49). Following release of these intramolecular salt bridges by the glycosaminoglycan, the hirudin-like sequence promotes interaction of HcII with thrombin exosite I. Thus, consideration of the HcII example and information on glycosaminoglycan binding to and activation of other serpins (47–51) suggest that oligosaccharide effector molecules utilize a variety of different mechanisms to activate serpins.

The Heparin Binding Site and Changes in the Affinity of Antithrombin for Heparin—Elucidation of the ATIII heparin binding site also provides a structural basis for understanding why there are differences in the affinity of different ATIII conformations for heparin. Rapid kinetic studies of heparin and pentasaccharide binding to ATIII have revealed a two-stage process consisting of an initial low affinity encounter reaction, followed by a protein conformational change and high affinity binding in the second step (21). According to the mechanism discussed above, an initial low affinity interaction with heparin occurs because heparin binding residues Lys125 and Arg129 (and perhaps Arg47) participate in intramolecular salt bridges in the native molecule (Fig. 4b). However, the strength of electrostatic interactions between these residues and negatively charged groups on heparin is predicted to increase as the native intramolecular salt bridges break in association with heparin binding, s123AhDEF movement, and the conformational transition to an activated, high heparin affinity molecule in the second stage of the binding reaction (Fig. 4, c and d). The dual roles of Lys125 and Arg129 in stabilizing the native structure and as heparin binding residues may explain why the second tight binding step is so closely linked to the protein conformational change that activates antithrombin. The 300-fold increase in affinity observed in the second step may also include contributions from conformational adjustments in the s123AhDEF fragment and/or changes in pentasaccharide geometry due to isomerization at the G iduronic acid unit (52).

Differences in heparin binding site configuration may also account for loss of heparin affinity after ATIII has interacted.
with a target enzyme. Heparin functions as a cofactor in the activation of antithrombin and dissociates from the inhibitory complex subsequent to promoting interaction of the bound and activated ATIII with a target enzyme. The cleaved form of ATIII also has reduced affinity for heparin. Comparison of the P14-inserted ATIII structure with those of cleaved bovine ATIII and latent human ATIII (data not shown) indicates that the configuration of heparin binding site residues changes upon deeper insertion of the reactive loop polypeptide into sheet A, as would occur during inhibitory complex formation and cleavage of the reactive loop. These changes in geometry are suggested to decrease the affinity of the inhibitory complex and cleaved ATIII for heparin and may reflect the overall protein conformational change that also causes certain epitope(s) to be absent from native and heparin bound ATIII, but exposed in inhibitory complexes of ATIII with thrombin and factor Xa, and in cleaved ATIII and binary complexes of ATIII with a reactive loop peptide (6). Consistent with decreased heparin affinity following inhibitory complex formation or ATIII cleavage, deeper insertion of the reactive loop polypeptide into sheet A has also recently been shown to dissipate electropositive surface potential present around the helix D region of P14-inserted ATIII (55).

Comparison with Expulsion Mechanism for ATIII Heparin Activation—We have experimentally identified helix D region basic residues that do and do not contribute to ATIII binding to heparin and analyzed our data with reference to the crystal structures of three different antithrombin conformations and two different native serpins. Our work suggests that heparin may activate antithrombin by breaking salt bridges that stabilize its native conformation. Weakening of the intramolecular salt bridges as a consequence of forming intermolecular salt bridges with heparin would allow s123AhDEFG movement and formation of conformationally activated molecule which is primed for reactive loop insertion and inhibiting coagulation enzymes. Implicit in our proposed mechanism is the presumption that heparin binds to a native molecule with a fully exposed reactive loop. This view differs fundamentally from proposed “expulsion” mechanisms for heparin-induced potentiation of antithrombin III (27, 56) which suggest that heparin binds to and converts an inactive, P14-inserted conformation to an activated, reactive-loop-out conformation by expelling the partially inserted reactive loop of the native molecule from the A-sheet.

According to the expulsion mechanism as proposed by van Boeckel and colleagues (27) heparin binding to Arg132, Lys125, and Lys136 elongates helix D, leading to P14 expulsion and reactive loop exposure. Our data disfavor this version of the expulsion mechanism based on the minor effects of the R132A, K133A, and K136A substitutions on ATIII heparin binding affinity. Meagher and colleagues (57) also found minor effects of R132M and K133M substitutions on pentasaccharide binding and heparin cofactor activation and also concluded that D helix extension is not the mechanism for transmission of conformational change from the heparin binding site to the reactive center region.

By studying heparin-induced intrinsic fluorescence changes of the ATIII P14 mutant S380W, Huntington and colleagues (56) obtained evidence for transfer of the P14 tryptophan from a less polar to a more polar environment upon binding of heparin. This observation was interpreted as proof that the P14 residue is initially buried in sheet A and then expelled into the solvent. However, an alternative interpretation of their data which would be consistent with a non-expulsion mechanism is also possible.

In the S380W study, the $\lambda_{\text{max}}$ of the fluorescence emission difference spectrum reports on the environment of the P14 tryptophan. The initial (heparin absent) $\lambda_{\text{max}}$ of 337 nm is intermediate between those of spectral form I (interior indoles near to polar groups emit at 330–332 nm) and spectral form II (indoles on a protein surface and in contact with bound water molecules of low mobility emit at 340–342 nm) (58). Our superpositions of P14-inserted ATIII with ovalbumin and native antichymotrypsin (described under “Materials and Methods” and shown in Fig. 3) predict the P14 residue of native S380W will be located at the base of the proximal stalk supporting the externalized reactive loop, with the indole ring nestled in a cavity formed by leucine 224 (s3A) and the hydrocarbon portions of lysines 226 (s3A), 228, 275 (s2B) and 136 (helix D-s2A turn). Thus, the environment of the P14 residue in the predicted structure of 5-stranded, reactive loop-out, native antithrombin is consistent with the observed 337 nm $\lambda_{\text{max}}$ in the absence of heparin.

In the S380W study, binding of heparin red shifted the difference spectrum $\lambda_{\text{max}}$ to 354 nm. This indicates that the P14 tryptophan moves to a surface location in contact with free water molecules when the heparin binding site is occupied. We propose that release of the P14 residue into solvent is caused by intramolecular salt bridge breakage and the s123AhDEFG shift which occur as a consequence of heparin binding to the site we have identified. Specifically, when s123AhDEFG moves away from $\beta$-sheet B and the reactive center end of the molecule (i.e. leftwards and toward the reader in Fig. 3), the shift of leucine 224 and lysine 226 in s3A and lysine 136 at the helix D-s2A turn will break up the hydrophobic cavity that encloses the P14 residue in native, 5-stranded antithrombin and promote its release into the solvent.

Proposed Structure of Heparin-activated ATIII—Thus, we propose that the activated form of antithrombin in the tight complex with heparin is one in which the intramolecular salt bridges have been broken and s123AhDEFG has shifted to release the P14 residue and “loosen” the reactive loop (but the reactive loop is still not yet incorporated as s4A). ATIII in the high affinity complex with heparin is primed for target proteinase inhibition, because s123AhDEFG has moved to a position similar to that seen in 6-stranded serpins and the proximal stalk of the reactive loop (containing the P14 residue) has been freed from its native position close to the surface of the molecule.

The structure we are proposing as the activated conformation is similar to P14-inserted antithrombin in that s123AhDEFG has shifted. However, it is distinct from P14-inserted ATIII in that the reactive loop has not yet entered the A-sheet. Thus, the activated structure in the tight complex with heparin can be thought of as a “precursor” to the P14-inserted structure observed in crystals of heterodimeric ATIII (12, 13, 54). Partial insertion of the reactive loop in P14-inserted ATIII may be related to interaction of more distal parts of the reactive loop with the latent molecule of the crystallized dimer.

Conclusion—We have identified the antithrombin III heparin binding site by alanine scanning mutagenesis of basic residues in the helix D region and proposed a mechanism for heparin activation of antithrombin III which is based on the observation that three heparin binding residues (Arg47, Lys125, and Arg129) are located at the interface between two fragments of serpin structure known to move relative to each other during serpin reactive loop insertion and inhibitory complex formation. Based on structural analysis of homologous native serpins, we hypothesize that native ATIII is stabilized by two or more heparin-sensitive cross-links and that the structural basis for heparin cofactor activity may be heparin release of these salt bridges, allowing movement of the s123AhDEFG fragment...
and generation of an activated species that is conformationally primed for reactive loop uptake by central β-sheet A and for inhibitory complex formation. This proposal makes specific predictions about the contributions that individual heparin binding residues make at each stage of heparin binding to and activation of ATIII and will be evaluated by investigating the behavior of appropriate mutants.

Acknowledgments—We thank I. Bjork and S. Olson for discussions and their insightful comments; D. Katz, D. Christianson, and L. Mourey for pre-release access to coordinates; and J. Fenton for thrombin.

REFERENCES

Identification of the Antithrombin III Heparin Binding Site
Eva Ersdal-Badju, Aiqin Lu, Yancheng Zuo, Veronique Picard and Susan Clark Bock

doi: 10.1074/jbc.272.31.19393

Access the most updated version of this article at http://www.jbc.org/content/272/31/19393

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 21 of which can be accessed free at http://www.jbc.org/content/272/31/19393.full.html#ref-list-1