Elaboration of P1 Arginine 393 Interaction with Underlying Glutamic Acid 255 Partially Activates Antithrombin III for Thrombin Inhibition but Not Factor Xa Inhibition

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The mechanism for heparin activation of antithrombin III has been postulated to involve disruption of interactions between its reactive loop P1 residue and Glu255 on the underlying protein surface. To test this hypothesis, the potential P1-constraining Arg→Glu255 hydrogen bond and ionic interactions were eliminated by converting Glu255 to alanine. E255A and wild-type ATIIIIs have identical reactive loop sequences (including the P1 and P14 residues), but differ in that Glu255-mediated, P1-constraining interactions with the underlying surface cannot form in the mutant. Relative to its wild-type parent, E255A had a 5-fold higher affinity for heparin and pentasaccharide. In the absence of cofactor, E255A exhibited a 5-fold activation of thrombin inhibition but no activation of factor Xa inhibition. Pentasaccharide addition elicited no further activation of thrombin inhibition but increased the factor Xa inhibition rate 100-fold. E255A heparin-dependent thrombin and factor Xa inhibition rates were 1000- and 2-fold faster, respectively, than pentasaccharide-catalyzed rates. Although “approximation” is the predominant factor in heparin activation of ATIII thrombin inhibition, and removal of the P1 constraint plays a distinct but minor role, the primary determinant for activation of factor Xa inhibition is the pentasaccharide-induced conformational change, with approximation making a further minor contribution, and removal of the P1 constraint playing no role at all.

Antithrombin III (ATIII) is a key inhibitor of blood coagulation enzymes and belongs to the serpin family of protease inhibitors whose members employ a common inhibition mechanism. However, the rate at which ATIII inhibits its target enzymes is abnormally low for a serpin, and activation by heparin or vascular wall heparan sulfate proteoglycans (HSPGs) is necessary for it to achieve the high inhibition rates characteristic of other serpins. The physiological importance of ATIII-heparin/HSPG interactions is supported by the occurrence of thrombosis in individuals carrying ATIII molecules with defects in heparin binding or activation, and by the clinical use of pharmaceutical heparin as an anticoagulant in patients with hereditary and acquired thrombosis.

Because of the extensive use of heparin as a pharmaceutical, the mechanism for heparin activation of ATIII anticoagulant activity has been extensively investigated. These studies show that dramatically increased rates of thrombin and factor Xa inhibition can be achieved upon binding of heparin. Heparin binding to antithrombin is a two-step process consisting of an initial low affinity interaction that induces a large scale protein conformational change, which leads to high affinity binding and ATIII activation (1). The rate enhancement for the inhibition of thrombin results from an approximation (bridging) mechanism in which long, high affinity heparin chains (≥18 sugars) facilitate a >1000-fold increased rate of association between thrombin and ATIII (2). In contrast, a specific anticoagulantly active pentasaccharide sequence of heparin is sufficient for accelerating the rate of factor Xa inhibition by ATIII. Binding of this pentasaccharide causes a protein conformational change and is associated with a 270-fold increased rate of factor Xa inhibition and a 1.7-fold increase in rate of inhibition of thrombin (3).

As a part of the effort to understand how heparin activates antithrombin III, it is important to determine why its basal rate of target enzyme inhibition is depressed compared with other serpins. The low rates of association between ATIII and its target enzymes have been attributed to problems with accessibility of its P1 residue, arginine 393. On the basis of comparisons of x-ray structures for native antithrombin (4, 5) and studies of a P14 serine-to-tryptophan substitution mutant (8) show that upon occupation of the pentasaccharide binding site, the P14 residue is expelled from the sheet A, lengthening the reactive loop. Expulsion of the P14 residue by pentasaccharide binding, P14 modification with fluorescein (9), or P14 substitution with glutamic acid (10) is associated with a 200–300-fold increase in the rate of association with factor Xa and a much smaller 2-fold increase in the rate of reaction with thrombin. Therefore, the P14 constraint on reactive loop length and mobility plays a significant role in depressing the native ATIII rate of factor Xa inhibition but makes a negligible contribution to the low rate of thrombin inhibition.

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#The abbreviations used are: ATIII, antithrombin III; HAH, full-length high affinity heparin; H5, heparin pentasaccharide; kXa, factor Xa; kApp, observed pseudo-first-order rate constant; kApp, apparent second-order association rate constant; kSt, dissociation equilibrium constant; FACE, fluorophore-assisted carbohydrate electrophoresis; SI, stoichiometry of inhibition; I, ionic strength; PEG, polyethylene glycol.

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Native ATIII recognition by target proteinases is additionally thought to be affected by a second reactive loop constraint, which is also specific to antithrombin compared with other serpins. The second constraint directly involves the arginine 393 P1 residue and causes it to face towards the body of the inhibitor rather than outwards toward the solvent in native ATIII (Fig. 1). As observed in crystal structures of native ATIII (4, 5), this constraint involves hydrogen- and ionic-bonding interactions between the P1 (Arg<sup>393</sup>) and P6<sup>6</sup> (Arg<sup>396</sup>) arginines of the reactive loop polypeptide and a glutamic acid residue (Glu<sup>255</sup>), which projects out from the C sheet in the body of the inhibitor. Although there has been some question as to whether the 393/399/255 constraint observed in the native and other ATIII structures (7, 11) could be a consequence of dimerization between two ATIII molecules in the unit cells of the crystals and the incorporation of the reactive loop polypeptide of one molecule into B-sheet C of the other molecule, the existence of stabilizing 393/399/255 interactions under physiological conditions is supported by solution studies of increased P1 accessibility to the enzyme peptidylarginine deiminase in the presence of heparin pentasaccharide (7, 12). Therefore, it has been proposed that a constraint on reactive site loop movement and P1 accessibility imposed by internal bonding with the side chain of arginine 393 contributes to the low inhibitory activity of native conformation ATIII and that release of this constraint is an important step in the activation of ATIII by heparin cofactor (5, 7, 12).

The role of the P1 constraint in heparin activation of ATIII has been previously addressed in several studies. Two studies utilizing fluorescent reporter groups attached at the P1 position concluded that heparin binding to antithrombin does not greatly alter solvent accessibility (13, 14) or motional freedom of the P1 side chain (14). Work with ATIII variants in which the P1 arginine 393 was replaced with a series of nonionic residues or histidine also imply that the P1 residue is similarly accessible to proteinases in the native and heparin-activated states (13). However, because of the critical role of the P1 residue in serpin target enzyme interactions, the approach of substituting the P1 amino acid in these studies produced a concomitant change in the target protease specificity, and it was not possible to directly address the role of the P1 constraint with respect to the normal physiological targets of ATIII, thrombin and factor Xa.

As described in this report, an alternative strategy for investigating the role of the P1 constraint in modulating ATIII anticoagulant activity involves alanine substitution of the negatively charged Glu<sup>253</sup> residue that projects from the serpin body and interacts with the P1 and P6<sup>6</sup> arginines. This approach has the advantages of maintaining the normal P1 arginine and wild-type reactive loop sequence and therefore permits investigation of the heparin cofactor mechanism to be conducted with respect to the normal physiological targets of ATIII. Using this approach we have quantitated the contribution of three different factors (the P1 and P14 reactive loop constraints and the approximation effect) to heparin cofactor activation of thrombin and factor Xa and have shown that at this level of resolution the detailed mechanisms for modulating ATIII inhibition of these enzymes are fundamentally different. Specifically, relief of the P1 constraint plays a minor role, relief of the P14 constraint plays no role, and approximation plays a major role in heparin modulation of ATIII thrombin inhibition. In contrast, relief of the P1 constraint plays no role, relief of the P14 constraint plays a major role, and approximation plays a minor role in the heparin modulation of factor Xa inhibition.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—The E255A mutation was made on a B-ATIII background (N135A) to reduce heparin binding heterogeneity and facilitate purification (15). PCR-based megaprimer mutagenesis was as previously described (16) using 5′-AGTACAGCGCCGCGCAAGTCC-3′ as the mutagenic nucleotide, 5′-GGGTGTCUAATAAGACCG-3′ (AT3. 708R) as the forward primer, and 5′-CTGCCCTCTATTTAATCCT-3′ (AT3.1282R) as the reverse primer. A mutant clone was identified by NcoI digestion. Following sequence verification of the entire 472-bp SacI–NsiI fragment, it was used to replace the corresponding portion of the human ATIII.N135A cDNA sequence in pFastbac1, followed by transposition and virus production in the Bac-to-Bac baculovirus expression system (Invitrogen).

**Recombinant ATIII Purification**—Supernatants from Sf9 Spodoptera frugiperda cells infected with baculovirus carrying the cDNAs for human ATIII.N135A or ATIII.N135A/E255A were harvested and cleared of cells and debris at 4–5 days postinfection, when the trypan blue viability of the host cells had dropped to ~80%. After passage through a 22-micron polyethersulfone membranes, supernatants were applied to 5-ml Hi-trap heparin columns (Amersham Biosciences) and eluted with a NaCl gradient. The column buffer was 1× PNE (20 mM sodium phosphate, pH 7.4, 0.1 mM EDTA, 0.1 M NaCl, 0.1% PEG 6000, pH 7.4, 0.1 M NaCl, 0.1% PEG 6000) and, concentrated by tangential ultrafiltration through 30,000 MWCO membranes (Vivaspin-20). The concentrated sample was applied to a 5-ml Econopak Q column (Bio-Rad) equilibrated with Q buffer, and the ATIII was eluted with a 45-ml gradient of 0–0.6 M NaCl in Q buffer. Peak activity fractions were pooled and dialyzed against 1× PNE and concentrated by ultrafiltration. Purity of heparin-containing fractions was asayed by SDS-PAGE electrophoresis. Protein concentration was determined from 280-nm absorbance using the molar extinction coefficient of plasma ATIII (17).

**High Affinity Heparin Purification—** 1 g samples of the heparin PZ fraction from porcine intestinal mucosa (Celsus) were resuspended in 15 ml of 0.05 M Tris-HCl, pH 7.4, 0.05 M NaCl and size-fractionated on a 30-cm human plasma ATIII-Sepharose column equilibrated with 0.1 M NaCl and a 30-cm Sephacryl HR-100 column (Amersham Biosciences). Heparin-containing fractions were identified by the Azure A method (18), and the size of heparins in each fraction determined by fluoroephore-assisted carbohydrate electrophoresis (FACE) (19) versus high affinity heparin (HAH) standards generously provided by Drs. S. Olson and I. Bjork. Fractions containing 20,000-Da heparins (range: 17,000–23,000 Da) from four Sephacryl columns were pooled and applied to a 30-ml human plasma ATIII-Sepharose column equilibrated with 0.05 M Tris-HCl, pH 7.4, 0.05 M NaCl. Heparins were eluted with 1 liter 0.05–0.3 M NaCl gradient at 45 ml/h. Azure A analysis of the fractions revealed the separation of non-absorbing, low affinity, and high affinity heparins from the column. Fractions containing high affinity heparin were pooled and concentrated and desalted using Centriprep 10 concentrators. Lophylized high affinity heparin was stored at ~70 °C. High affinity heparin was concentrated by stoichiometric fluorescence titrations versus human ATIII. The material referred to as heparin in these studies is high affinity heparin prepared as above.

**Experimental Conditions for Inhibition and Binding Studies—** All experiments were conducted at 25 °C in 20 mM sodium phosphate, pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 0.1% PEG 6000 (PNE-PEG). In the absence of added salt, the affinity of heparin for PNE-PEG increased to 15 mg/ml at pH 7.4. NaCl was added to achieve the higher ionic strength in the heparin binding studies.

**Thrombin and Factor Xa Inhibition Stoichiometry—** Stoichiometries for N135A and N135A/E225A inhibition of thrombin and factor Xa in the absence and presence of pentasaccharide or heparin were determined by titrating active site-titrated thrombin and factor Xa with the above heparins (Table I) and measuring enzymatic rates. Pentasaccharide and active site-titrated enzymes were kindly provided by Dr. Steve Olson, and high affinity heparin was prepared as described above. When included, heparin and pentasaccharide were present at 2 and 2.5 µM, respectively, corresponding to a 2-fold molar excess over the highest ATIII concentration. For determination of inhibition stoichiometries, 12-µl reactions containing 0–1000 nM antithrombin and 400 nM target enzyme in PNE-PEG were incubated at 25 °C for >10 times the half-life.
of inhibition of the combination being tested. Then 5 μl of the reaction mixture were subsampled into 195 μl of PNE-PEG, and the remaining 7 μl were frozen. 60-μl aliquots of the diluted reaction mix were transferred to a 96-well plate. Residual enzyme activity was measured by adding 100 μl of 1.5 mM thrombin substrate S-2238 or factor Xa substrate S-2765 (Chromogenix) and recording the initial hydrolysis rate at 405 nm. Percent residual enzyme activity was plotted versus the I/E (inhibitor/enzyme) ratio. Stoichiometry of inhibition (SI) was determined by extrapolating the plot to the molar ratio corresponding to complete proteinase inhibition. In addition to functional monitoring of residual thrombin and factor Xa inhibitory activity, the types of ATIII complexes and products formed at different I/E ratios were determined by analysis of the remaining 7 μl of the reaction mix on 12% polyacrylamide Laemmli gels under non-reducing conditions.

**Thrombin and Factor Xa Inhibition by N135A and E255A/N135A—** Reactions for the measurement of progressive activity were set up under pseudo-first-order conditions and contained ATIII and target enzymes in molar ratios ranging from 10:1 to 30:1. ATIIIs and 10 nM thrombin or Xa were reacted in individual microplate wells in a total volume of 80 μl. The buffer was PNE-PEG plus 50 μg/ml polybrene for thrombin wells and PNE-PEG for Xa wells. Following incubations of 5–40 min, residual thrombin and Xa activity were measured by adding 100 μl of 0.15 mM S2238 or S2765, respectively. Observed pseudo-first-order rate constants, $k_{obs}$, were obtained from the negative slope of a plot of ln(residual enzyme activity) versus time of enzyme and inhibitor co-incubation. Second-order rate constants ($k_{app}$) were obtained by dividing the observed pseudo-first-order rate constants ($k_{obs}$) by the ATIII concentration. With several modifications the same method was used to measure the inhibition of thrombin and Xa by N135A and E255A/N135A in the presence of various concentrations of pentasaccharide and heparin. ATIII target enzyme co-incubation times ranged from 0.5 to 40 min, and 50 μg/ml polybrene was included with both chromogenic substrates to quench the cofactor-dependent reactions. Observed pseudo-first-order rate constants were calculated for each heparin (0.5–10 nM) concentration from the slope of ln(residual enzyme activity) versus time of enzyme and inhibitor or pentasaccharide-ATIII complex ($k_{app}$ or $k_{obs}$) from Equation 2 (20) using the $K_d$ for heparin binding at the ionic strength of the reaction (0.15).

$$k_{obs} = \frac{(K_d[H])[AT]}{[AT] + [K_d]} + K_d[AT]$$  \hspace{1cm} (Eq. 1)  

$k_{app}$ values were corrected for the partition along the substrate pathway by multiplying the mean of 2–3 measurements by the SI value obtained for the same ATIII-target enzyme-heparin combination.

**Heparin Binding Studies—** Stoichiometries and equilibrium dissociation constants ($K_d$) for pentasaccharide and heparin binding to ATIII were determined by monitoring the tryptophan fluorescence enhancement, which accompanies the cofactor-binding interaction (20). Titrations were performed at 25 °C with a PC1 spectrofluorometer (ISS, Champaign, IL) using an excitation wavelength of 280 nm and emission wavelength of 340 nm. Solutions for heparin titrations contained 20 mM sodium phosphate, 0.1 mM EDTA, 0.1% (w/v) polyethylene glycol 6000 (pH 7.4) plus NaCl to adjust the ionic strength. Fluorescence intensities were corrected for sample volume increases during titration, and the data were fit to Equation 2 by nonlinear regression curve fitting with Graphpad Prism software.

$$\Delta F = \Delta F_{max} \times \left(\frac{[AT] + [H + K_d]}{K_d} - \left(\frac{[AT] + [H + K_d]}{[AT] + [H]}\right)^2 - 4 \times [AT] \times [H]\right)^{1/2}$$ \hspace{1cm} (Eq. 2)  

In Equation 2, $\Delta F$ is the fractional fluorescence change at a given heparin or pentasaccharide concentration, H is the heparin or pentasaccharide concentration, $\Delta F_{max}$ is the maximum fluorescence enhancement.

**Results**

**Expression and Purification—** Recombinant E255A/N135A human antithrombin III was expressed in the baculovirus system as previously described (15). The substitution of interest was produced on an N135A β-antithrombin isofrom background to eliminate N-glycosylation and heparin binding heterogeneity resulting from the production of two glycoforms when the wild-type sequence, asparagine-proline-serine, is present at residues 135–137 (21).

E255A/N135A was purified from supernatants of infected S9 cells by two rounds of affinity chromatography on heparin-Sepharose. A second pass over immobilized heparin was necessary to remove latent ATIII molecules, which represent a substantial fraction of the antithrombin in E255A/N135A supernatants. Purified E255A/N135A and its N135A parent co-migrated on SDS-polyacrylamide gels and were >95% pure.

Stoichiometries for E255A/N135A and N135A inhibition of thrombin and factor Xa were measured in the absence and presence of cofactor (Table I). The SI values obtained for E255A/N135A and N135A inhibition of thrombin and factor Xa in the absence of heparin were in good agreement with each other, and with heparin binding stoichiometries of 0.65–0.8, obtained by fluorescence titration. In the presence of heparin, significantly more E255A was required for complete inhibition of both thrombin and factor Xa, as was also observed for the parent N135A molecule, and is well established for plasma-derived antithrombin (22). The similarity of the mutant and parent data indicates that E255A/N135A is correctly folded and that the mutation does not alter the basic mechanism or efficiency of target proteinase inhibition.

**Heparin Binding—** E255A/N135A eluted from immobilized heparin at a higher ionic strength (2.8 mM NaCl) than its N135A parent (2.1 mM NaCl). The magnitude of the increased E255A affinity for heparin was determined by measurement of the dissociation equilibrium constants for binding to pentasaccharide and full-length high affinity heparin (Table II). Fluores-
To better understand the inhibition of thrombin and factor Xa, we determined the association rate, activity, and corrected for flux across the substrate pathway. This was done to ensure accurate measurement of the inhibition. The N135A parent was 93 nM (in good agreement with previous measurements, Refs. 15 and 23), whereas that of the N135A variant was 18 nM. This suggests a 5-fold increase in antithrombin affinity for both heparin and pentasaccharide.

The kinetic loss of target proteinase was measured at pH 7.4, 25°C, and 0.15 ionic strength. Apparent second-order rate constants, \( k_{\text{app}} \), were determined from the kinetic loss of target proteinase and corrected for flux across the substrate pathway by multiplying the apparent rate constant by SI values reported in Table I. Values are mean ± S.E. for three measurements, except as noted.

|                    | Thrombin (\( k_a \))   | Factor Xa (\( k_a \)) |
|--------------------|------------------------|-----------------------|
|                    | uncat +H5 +HAH         | uncat +H5 +HAH        |
| N135A              | \( 4.0 \pm 0.1 \times 10^4 \) | \( 3.6 \pm 0.1 \times 10^8 \) |
| E255A/N135A        | \( 2.1 \pm 0.3 \times 10^4 \) | \( 3.5 \pm 0.7 \times 10^8 \) |

* Two measurements.

**Fig. 1. ATIII P1 and P14 constraints.** Reactive loop end of the inhibitory molecule of native human antithrombin (2ant.pdb, Ichain) (26) illustrating stabilizing interactions between arginine 393 (P1) and P6' arginine 399 in the reactive loop polypeptide and glutamic acid 255 projecting out from the body of the inhibitor and internalized location of the P14 residue (S380).

**Fig. 2. Relative contributions of release of the P1 and P14 constraints and the approximation effect to activation of anti-thrombin III thrombin and Xa inhibition.** Cartoons in the left column schematically depict the status of the P1 and P14 constraints in native wild-type ATIII (a) and the E255A variant without cofactor (b) or with bound pentasaccharide (c) or full-length heparin (d). Arrows signify relative rates of inhibition of thrombin (center column) and factor Xa (right column) with the wild-type and E255A antithrombins under different conditions. The areas of the shafts of the arrows are proportional to the association rates reported in Table III. See “Discussion” for details.

N135A parent, but there was no change in the rate of factor Xa inhibition. Thus, the native conformation of the E255A variant is partially activated for thrombin inhibition, but not factor Xa inhibition.

Patterns of heparin cofactor activation for E255A/N135A and N135A inhibition of thrombin differed. As has been previously demonstrated (23), patterns are different under different conditions.

Patterns of heparin cofactor activation for E255A/N135A and N135A inhibition of thrombin differed. As has been previously demonstrated (23), patterns are different under different conditions.
The P1 arginine (393) and arginine 399 in the reactive loop of ATIII interact with glutamic acid residue 255, which underlies them and extends out from the body of the molecule (Fig. 1). The goal of this work was to investigate the role of the 393/399/255 reactive loop-stabilizing constraint on antithrombin heparin cofactor activation. Because the P1 arginine and the reactive loop polypeptide are central to the interaction of ATIII with its target proteinases, we chose to avoid specificity issues associated with the changing of reactive loop residues and to make an E255A substitution mutant that is altered in the component of the interaction originating on the serpin body side of the interaction. This allowed us to study the effect of 393/399/255 reactive loop–serpin body stabilization in the context of antithrombin’s normal physiological targets, thrombin and factor Xa.

Fig. 2 summarizes the effects of the P1 and P14 reactive loop constraints and pentasaccharide and full-length heparin binding on rates of ATIII thrombin and factor Xa inhibition. In this figure, the arrows represent relative rates of thrombin or factor Xa inhibition by ATIII variants with wild-type or E255A reactive site regions in the absence and presence of pentasaccharide or full-length high affinity heparin. The areas of the shafts of the arrows are proportional to the magnitude of the association rate for each ATIII/heparin/target enzyme combination. A comparison of rows a and b shows that selective removal of the 393/399/255 constraint increases the rate of association with thrombin 5-fold but has no effect on the rate of factor Xa inhibition. Comparison of rows b and c indicates that upon subsequent release of the P14 constraint by binding of pentasaccharide, the rate of factor Xa inhibition increases 100-fold, whereas there is no additional increase in the rate of reaction with thrombin over what was achieved by removal of the 393/399/255 constraint. Together these observations indicate that thrombin reacts more efficiently with P1-accessible versus P1-inaccessible (393/399/255-constrained) native ATIII conformations. In contrast, the natural (13) or mutationally favored (E255A) equilibrium between P1-accessible versus P1-inaccessible native ATIII conformations is of no consequence with respect to facilitating factor Xa interactions. Further release of the P14 constraint by occupancy of the pentasaccharide binding site is required to increase the rate of the factor Xa reaction. Therefore, on the basis of these observations we suggest that under progressive inhibition conditions in the absence of heparin, the key initial interactions of the antithrombin reactive loop with thrombin are localized in the P1 region, whereas the key initial interactions with factor Xa take place in a region of the reactive loop that is freed by P14 expulsion, or, as previously proposed by others (13, 14), in an ATIII factor Xa binding exosite that is exposed by the conformational change.

Comparison of Fig. 2, rows c and d indicates that extension of the pentasaccharide with additional oligosaccharide residues increases the rate of thrombin inhibition by the E255A variant 1000-fold but has only a modest effect (2-fold) on further accelerating the rate of factor Xa inhibition. The magnitudes of the accelerating effects for full-length heparin versus pentasaccharide are similar to what are observed for the N135A parent molecule and plasma-derived antithrombin (Table III and Ref. 23).

Together the information contained in Table III and Fig. 2 reveals the relative contributions of (i) the reactive loop P1–393/399/255 constraint, (ii) the reactive loop P14 constraint, and (iii) approximation, to heparin activation of antithrombin III thrombin and factor Xa inhibition. With respect to thrombin inhibition, release of the P1 (393/399/255) constraint makes a modest contribution to accelerating the reaction with thrombin, whereas release of the P14 constraint plays no role at all. In the case of thrombin, it is approximation of the inhibitor and thrombin by full-length heparin that is overwhelmingly responsible for the activation of thrombin inhibition. In contrast, the P1 constraint plays no role and approximation plays a relatively minor role, in heparin cofactor inhibition of factor Xa. Instead, it is the protein conformational change associated with occupancy of the pentasaccharide binding site that drives heparin activation of ATIII factor Xa inhibition.

Comparisons of full-length high affinity heparin and pentasaccharide binding to E255A/N135A and N135A are presented in Table II and provide information about protein conformational effects of the P1 constraint. The increased affinity of E255A/N135A for pentasaccharide and full-length high affinity heparin suggests that the 393/399/255 reactive loop constraint is responsible for stabilizing a conformation of the inhibitor with low, native-level affinity for heparin. In addition, the data provide further evidence for structural linkage between the reactive loop and the pentasaccharide binding site on helix D. Removal of the 393/399/255 constraint in the E255A/N135A mutant was associated with 5-fold increases in the affinities for full-length heparin and pentasaccharide. Similar increases in full-length heparin binding affinity have been observed for natural and recombinant ATIII P1 substitutions (13, 24). Together the increased full-length heparin affinities of the E255A/N135A and P1 substitution mutants show that release of the 393/399/255 constraint is associated with a protein conformational change involving the extended binding site for full-length heparin. However, the 5-fold increased pentasaccharide affinity reported here for the E255A/N135A variant relative to its N135A parent more precisely demonstrates conformational linkage between the reactive loop and the pentasaccharide binding site. This finding complements previous observations that have demonstrated the same conformational linkage from the opposite point of view, i.e. based on occupancy of the pentasaccharide binding site affecting the presentation and properties of the reactive loop (25).

In summary, work on an antithrombin E255A mutant that eliminates the P1 reactive loop constraint shows that the mechanisms for heparin cofactor acceleration of thrombin and factor Xa inhibition are distinct and complex, and that activation of this physiologically important anticoagulant protein involves more than the simple increased exposure of its P1 arginine.

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