Localization of an Antithrombin Exosite That Promotes Rapid Inhibition of Factors Xa and IXa Dependent on Heparin Activation of the Serpin*

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We have previously shown that exosites in antithrombin outside the P6–P3 reactive loop region become available upon heparin activation to promote rapid inhibition of the target proteases, factor Xa and factor IXa. To identify these exosites, we prepared six antithrombin-α,2-proteinase inhibitor chimeras in which antithrombin residues 224–286 and 310–322 that circumscribe a region surrounding the reactive loop on the inhibitor surface were replaced in 10–16-residue segments with the homologous segments of α,2-proteinase inhibitor. All chimeras bound heparin with a high affinity similar to wild-type, underwent heparin-induced fluorescence changes indicative of normal conformational activation, and were able to form SDS-stable complexes with thrombin, factor Xa, and factor IXa and inhibit these proteases with stoichiometries minimally altered from those of wild-type antithrombin. With only one exception, conformational activation of the chimeras with a heparin pentasaccharide resulted in normal —100–300-fold enhancements in reactivity with factor Xa and factor IXa. The exception was the chimera in which residues 246–258 were replaced, corresponding to strand 3 of β-sheet C, which showed little or no enhancement of its reactivity with these proteases following pentasaccharide activation. By contrast, all chimeras including the strand 3C chimera showed essentially wild-type reactivities with thrombin after pentasaccharide activation as well as normal full-length heparin enhancements in reactivity with all proteases due to heparin bridging. These findings suggest that antithrombin exosites responsible for enhancing the rates of factor Xa and factor IXa inhibition in the conformationally activated inhibitor lie in strand 3 of β-sheet C of the serpin.

Antithrombin is the major serpin family regulator of blood clotting proteases in plasma (1–3). This essential anticoagulant protein inhibits clotting proteases, mainly thrombin, factor Xa, and factor IXa, by forming stable equimolar complexes with the enzymes through an unusual mechanism, which is shared by other serpin family protease inhibitors (1, 4, 5). In this mechanism, the protease initially recognizes an exposed reactive

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Proteases—Human α-thrombin was prepared by first purifying protrombin from plasma and then activating the zymogen and purifying the enzyme as described (33, 34). Factor Xa (predominantly α-form), factor IX and factor Xla were purchased from Enzyme Research Laboratories (South Bend, IN). Factor IXα was obtained by activation of the zymogen with factor Xla as described (5).

Heparins—A synthetic hepamin pentasaccharide corresponding to the antithrombin binding pentasaccharide in heparin (11) was generously provided by Maurice Petitou (Sanofi Recherche, Toulouse, France). A full-length hepamin with reduced polydispersity that contained the pentasaccharide and had an average chain length of ~50 saccharides was isolated from commercial hepamin by size and antithrombin affinity fractionation as previously described (35). Concentrations of hepamin were titrated by stoichiometric titrations of antithrombin with the saccharides monitored by tryptophan fluorescence changes accompanying the interaction as in previous studies (13, 35).

SDS and Native PAGE—The purity of recombinant antithrombins was verified by reducing or nonreducing SDS-PAGE on 10% acrylamide gels using the Laemmli discontinuous buffer system (36). The ability of the recombinant inhibitors to form SDS-stable complexes with thrombin and factor Xa was evaluated by reacting 1.6 μM recombinant antithrombin and 0.8 μM protease for 15–18 h or in some cases for 1 h. Native PAGE was conducted with the Laemmli buffer system under nondenaturing conditions.

Experimental Conditions—All experiments were conducted at 25 °C in 20 mM potassium phosphate, 0.1 mM EDTA or 100 mM Hepes, 5 mM CaCl₂, and then chromatographing the concentrate on a 5-ml HiTrap heparin-Sepharose column (Amersham Biosciences) with an extinction coefficient of 37,700 M⁻¹ cm⁻¹. The wild-type protein was measured from the absorbance at 280 nm.

Construction, Expression, and Purification of Wild Type and Variant Antithrombins—Recombinant antithrombins were constructed on an N135Q background by ligating the cDNAs encoding the antithrombins into the pFastBac1 vector from the Bac-to-Bac baculovirus expression system (Invitrogen). Mutations were introduced into the N135Q antithrombin cDNA by the splicing-overlapping extension method (29) utilizing the Fλ DNA polymerase (Stratagene, La Jolla, CA). Briefly, two oligonucleotides encoding the changes for overlapping 5′-end and 3′-end segments of the mutated region in sense and antisense strands and extending outside of the mutated region were annealed to the antithrombin cDNA along with complementary strand primers and extended in separate PCRs. The PCR products of these reactions were then annealed through the overlap region and extended in a third PCR to generate a double-stranded antithrombin cDNA containing the desired mutations. Oligonucleotides for introducing the mutations were synthesized by Integrated DNA Technologies (Coraville, IA). All mutations were confirmed by DNA sequencing. Recombinant proteins were expressed in insect cells after infection with baculovirus containing the inserted wild-type or variant antithrombin cDNA as described previously (30, 31). Expression levels in the extracellular medium were estimated by Western blotting (30). Recombinant antithrombins were purified by first concentrating the medium by ultrafiltration (Millipore Corp., Bedford, MA) and then chromatographing the concentrate on a 5-ml HiTrap heparin-Sepharose column (Amersham Biosciences) with NaCl gradient elution as in previous studies (30). The concentration of the wild-type protein was measured from the absorbance at 280 nm using an extinction coefficient of 37,700 M⁻¹ cm⁻¹ (32). Concentrations of antithrombin variants were determined using the Micro BCA Protein Assay (Pierce) with wild-type antithrombin as a standard.

The abbreviations used are: α1,PI, α₁-proteinase inhibitor; AT, antithrombin.
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Amino acid substitutions in the antithrombin-a1PI chimeras

Shown are the linear sequences of human antithrombin, numbered based on their position in the serpin, which were replaced with the homologous sequences in human a1PI, shown below the antithrombin sequences, to generate the six chimeras. Residues that are identical in the two serpin sequences are boxed, residues >70% conserved in the 13 known antithrombin sequences (47) are in boldface type, and residues that are buried in the x-ray structure of antithrombin are in italic type. Secondary structural regions are underlined.

| Residue | a1PI | bH |
|---------|------|----|
| 224     | AT   | L  |
| 234     | W    | K  |
| 235     | K    | W  |
| 245     | W    | E  |
| 246     | M    | T  |
| 259     | K    | T  |
| 275     | K    | L  |
| 301     | E    | L  |
| 322     | M    | P  |

Design of the Antithrombin-a1PI Chimeras—We previously showed that conformational activation of antithrombin by heparin generates new interaction exosites outside the serpin reactive loop, which enhance the inhibitor’s reactivity with the target proteases, factor Xa and factor IXa (5, 22, 25). In order to map these exosites, we constructed six chimeric variants in which linear segments of antithrombin consisting of 10–16 residues were replaced with the homologous sequences from the prototypic serpin, a1PI. The replaced segments corresponded to regions of the inhibitor that circumscribe the reactive protease binding loop and that involve secondary structural elements lying within 20 Å of the principal specificity-determining residue of the reactive loop, the P1 arginine 393 residue (20). Examination of the structures of known factor Xa-proteinase inhibitor complex showed that the 20-Å distance would encompass the “reach” of the surface loops of factor Xa surrounding the enzyme active site, which were expected to interact with the putative exosite of antithrombin (38, 39). The replaced segments of antithrombin included residues 224–286 and 310–322, corresponding to six strands of β-sheets B and C and the loops connecting these strands (Table I). This region was subdivided by secondary structural units into (i) the C terminus of strand 3A, the strand 3A-helix F1 loop, and helix F1 (residues 224–234), (ii) strand 4C and the strand 4C-strand 3C loop (residues 235–245), (iii) strand 3C (residues 246–258), (iv) strand 1B, the strand 1B-strand 2B loop, and strand 2B (residues 259–274), (v) the strand 2B-strand 3B loop and strand 3B (residues 275–286), and (vi) the C terminus of helix H, the helix H-strand 2C loop, and strand 2C (residues 310–322) (Fig. 1). The chimeras retained those antithrombin residues that are highly conserved in all serpins including a1PI, these corresponding in most cases to hydrophobic core residues critical for serpin structure (28). In order to avoid other possible perturbations of antithrombin structure resulting from the a1PI sequence substitutions, the disulfide-bonded cysteine in strand 3C of antithrombin was retained, a serine substitution was made for the free cysteine in the strand 1B-strand 2B loop of a1PI, the single residue insertion in the same a1PI loop was not included in the antithrombin chimeras in which this region was replaced, and the single residue insertion in the antithrombin strand 2B-strand 3B loop was maintained by insert-

factor Xa. Reactions of free antithrombins and those reactions of antithrombin-pentasaccharide complexes in which stoichiometric heparin concentrations were employed with proteases were monitored by quenching reactions after varying times with 0.9–0.95 ml of 100–300 μM chromogenic or 100 μM fluorogenic substrate containing 50 μg/ml Polybrene to neutralize heparin when present. Residual protease activity was measured from the initial rate of substrate hydrolysis as described above. The observed pseudo-first-order rate constants (kobs) for the inhibition reactions were obtained by fitting the loss of enzyme activity to a single exponential decay function by nonlinear regression analysis (5, 37). The second-order rate constants for free antithrombin reactions were calculated by dividing kobs by the antithrombin concentration. Rate constants for heparin-catalyzed reactions were obtained by dividing kobs by the concentration of antithrombin-heparin complex calculated from measured Kd values for the interaction after correcting for any contribution of the free antithrombin reaction.

The kinetics of inhibition of thrombin, factor Xa, and factor IXa by recombinant antithrombins in the presence of catalytic levels of heparin were analyzed as a function of the heparin concentration for a fixed reaction time. Thrombin reaction times were 18 s, factor Xa times were 2–5 min, and factor IXa times were 1–2 min. The decrease in protease activity as a function of increases in heparin concentration was fit by a single exponential function based on the following equation (37).

\[ t = \frac{[P]_0 \times \exp(-k_{\text{uncat}} \times [AT]_0 \times t)}{k_H \times ([AT]_0/K_{\text{AT,H}} + [AT]_0) \times t + K_{\text{AT,H}} \times ([AT]_0/K_{\text{AT,H}} + [AT]_0) \times t} \] (Eq. 1)

In this equation, [P]0 and [P] denote protease concentrations at time 0 and time t, [AT]0 and [H]0 are antithrombin and heparin concentrations, kuncat and kH are the second-order rate constants for free antithrombin and antithrombin-heparin complex reactions with protease, and KAT,H is the dissociation constant for the antithrombin-heparin interaction. Rearranging this equation shows that the inhibition of protease will decay exponentially as a function of the heparin concentration when the reaction time is fixed.

\[ t = \frac{[P]_0 \times \exp(-k_{\text{uncat}} \times [AT]_0 \times t)}{k_H \times ([AT]_0/K_{\text{AT,H}} + [AT]_0) \times t} \] (Eq. 2)

The intercept of the exponential at zero heparin concentration in this case is equal to [P]0 \times \exp(-k_{\text{uncat}} \times [AT]_0 \times t), and the exponential constant is equal to kH \times ([AT]_0/K_{\text{AT,H}} + [AT]_0) \times t. The second-order inhibition rate constant for the heparin-catalyzed reaction is thus obtained by dividing the fitted exponential constant by the fixed reaction time and by the factor ([AT]_0/K_{\text{AT,H}} + [AT]_0), representing the fraction of antithrombin complexed with heparin. This analysis provides a robust determination of the association rate constant and validation of the second-order kinetic behavior over a range of antithrombin-heparin complex concentrations with minimal expenditure of reagents.

RESULTS

Design of the Antithrombin-a1PI Chimeras—We previously showed that conformational activation of antithrombin by heparin generates new interaction exosites outside the serpin reactive loop, which enhance the inhibitor’s reactivity with the target proteases, factor Xa and factor IXa (5, 22, 25). In order to map these exosites, we constructed six chimeric variants in which linear segments of antithrombin consisting of 10–16 residues were replaced with the homologous sequences from the prototypic serpin, a1PI. The replaced segments corresponded to regions of the inhibitor that circumscribe the reactive protease binding loop and that involve secondary structural elements lying within 20 Å of the principal specificity-determining residue of the reactive loop, the P1 arginine 393 residue (20). Examination of the structures of known factor Xa-proteinase inhibitor complex showed that the 20-Å distance would encompass the “reach” of the surface loops of factor Xa surrounding the enzyme active site, which were expected to interact with the putative exosite of antithrombin (38, 39). The replaced segments of antithrombin included residues 224–286 and 310–322, corresponding to six strands of β-sheets B and C and the loops connecting these strands (Table I). This region was subdivided by secondary structural units into (i) the C terminus of strand 3A, the strand 3A-helix F1 loop, and helix F1 (residues 224–234), (ii) strand 4C and the strand 4C-strand 3C loop (residues 235–245), (iii) strand 3C (residues 246–258), (iv) strand 1B, the strand 1B-strand 2B loop, and strand 2B (residues 259–274), (v) the strand 2B-strand 3B loop and strand 3B (residues 275–286), and (vi) the C terminus of helix H, the helix H-strand 2C loop, and strand 2C (residues 310–322) (Fig. 1). The chimeras retained those antithrombin residues that are highly conserved in all serpins including a1PI, these corresponding in most cases to hydrophobic core residues critical for serpin structure (28). In order to avoid other possible perturbations of antithrombin structure resulting from the a1PI sequence substitutions, the disulfide-bonded cysteine in strand 3C of antithrombin was retained, a serine substitution was made for the free cysteine in the strand 1B-strand 2B loop of a1PI, the single residue insertion in the same a1PI loop was not included in the antithrombin chimeras in which this region was replaced, and the single residue insertion in the antithrombin strand 2B-strand 3B loop was maintained by insert-
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Expression, Purification, and Inhibitory Activity of the Chimeras—All chimeras were expressed in yields comparable with the wild-type serpin and could be purified by heparin-agarose chromatography. Each of the variants retained high affinity for heparin-agarose similar to that of the wild-type inhibitor, as indicated from their elution from the matrix between 2 and 3 M NaCl. However, a significant fraction of the strand 3B chimera detected in the expression medium by Western blotting eluted at low salt concentration and was nonfunctional, presumably due to misfolding, since the functional protein eluting at high salt appeared to be stable. Each of the chimeras was highly purified after the single chromatography step as judged from the appearance of one major protein band on SDS-PAGE (Fig. 2). Further purification by Mono Q chromatography (42) did not affect the measured properties of the variants. Native PAGE analysis of the chimeras (not shown) showed single bands with no evidence of polymers and electrophoretic mobilities altered somewhat from wild-type in keeping with the changes in charged residues (Table I). All chimeras formed SDS-stable high molecular weight complexes with thrombin and factor Xa that were visible by SDS-PAGE analysis (shown for the factor Xa complexes in Fig. 2), indicating that the amino acid substitutions made in the antithrombin chimeras had not significantly affected the ability to inhibit the principal target proteases of the serpin. The measured stoichiometries for the reactions of wild-type and chimeric antithrombins with thrombin ranged between 1.0 and 1.4 mol of inhibitor/mol of thrombin, suggesting the presence of some nonfunctional, presumably latent, inhibitor in the preparations (~10–40%) as is typical of recombinant antithrombin preparations (42). This was confirmed by analysis of heparin binding stoichiometries (see below). Functional inhibitor concentrations were employed in subsequent measurements of inhibition rate constants.

Heparin Binding and Activation of the Chimeras—The effect of the α1PI sequence substitutions on heparin binding and conformational activation of the chimeric antithrombins was quantitatively analyzed by equilibrium binding titrations monitored from changes in intrinsic protein fluorescence (Fig. 3 and Table II). All chimeras underwent saturable increases in fluorescence upon titration with the specific heparin pentasaccharide, which were similar to those of the wild-type protein. Analysis of these titrations showed that the chimeras differed only slightly from the wild-type protein in heparin binding stoichiometry and heparin affinity (at most ~3-fold difference in K_D from the wild-type inhibitor). The similar stoichiometries verified that the chimeras were mostly or fully functional in binding heparin, and the observed differences from the wild-type protein paralleled those found in the thrombin inhibition titrations. Interestingly, the strand 3C chimera appeared to have a higher heparin affinity than wild-type. This finding was verifiable from titrations at a higher ionic strength of 0.35, where more accurately determined K_D values of 12 ± 3 and 80 ± 14 nM (average from two titrations ± range) were measured for chimeras and wild-type inhibitors, respectively, and from the elution of the strand 3C chimera from heparin-agarose at a somewhat higher NaCl concentration (2.9–3 M) than that for elution of the wild-type protein (2.5 M). All chimeras had their fluorescence maximally enhanced by heparin to an extent (34–58%) similar to that of the wild-type inhibitor (43%), indicating that all of the chimeras underwent essentially normal conformational activation by heparin (Table II).

Reactivity of the Chimeras with Proteases—The effects of the α1PI substitutions in the chimeric antithrombins on their reactivity with thrombin, factor Xa, and factor IXa were analyzed in the absence and presence of heparin by monitoring the

Fig. 1. Location in the antithrombin structure of linear segments substituted in the antithrombin-α1PI chimeras. The ribbon depictions of the antithrombin-heparin pentasaccharide complex structure (Protein Data Bank 1E03) show the regions substituted with homologous α1PI sequences in the six engineered antithrombin chimeras as different colors. A shows the entire molecule viewed from one side of the reactive loop, and B provides a close-up of just the altered regions viewed from the top of the reactive loop. Regions replaced are residues 224–234 (yellow), residues 235–245 (orange), residues 246–258 (red), residues 259–274 (green), residues 275–286 (purple), and residues 310–322 (cyan). The reactive loop residues 378–405 are shown in blue with the P6–P3′ residue side chains represented as sticks. The side chains of Tyr-253, Glu-255, and Lys-257 residues in strand 3C that are conserved in all known antithrombin sequences and the bound heparin pentasaccharide (A) are shown as sticks.
kinetics of protease inhibition under pseudo-first-order conditions as in past studies (5, 37). Because of the limited amounts of functional s3B chimera, kinetic analyses were only done with thrombin and factor Xa. The reactivity of all but the strand 2C and strand 3C chimeras with the three proteases was decreased significantly from that of the wild-type inhibitor in the absence of heparin (i.e. 5–7-fold with factor Xa, 20–30-fold with factor IXa, and up to ~100-fold with thrombin) (Fig. 4). The strand 2C and 3C chimeras showed normal reactivities with all proteases except for the reaction of the strand 3C chimera with thrombin, which was 20-fold faster than the wild-type inhibitor reaction. The polycation, Polybrene, had no effect on the uncatalyzed reactions of the antithrombin chimeras with thrombin, indicating that true uncatalyzed rate constants unaffected by contaminating heparin had been measured.

Heparin pentasaccharide activation of the chimeras normalized all of the rate constants for the reactions with thrombin to values at most ~3-fold different from that of the similarly activated wild-type inhibitor (Fig. 4). This was associated with substantial pentasaccharide enhancements in chimera reactivity with thrombin for several chimeras and remarkably a loss in reactivity in the case of the strand 3C chimera. Significantly, all of the chimeras except for one showed nearly normal enhancements in reactivity toward factor Xa and factor IXa upon pentasaccharide activation (i.e. 80–190-fold with factor Xa and 200–300-fold with factor IXa). The striking exception was the strand 3C chimera, which showed a drastically impaired enhancement in reactivity toward both factors Xa and IXa upon pentasaccharide activation. The pentasaccharide thus increased the strand 3C chimera’s reactivity ~2-fold toward factor Xa, equal to ~2% of the wild-type inhibitor enhancement, and decreased its reactivity toward factor IXa ~40%.

As with pentasaccharide activation, a full-length bridging heparin normalized the reactivities of all chimeras with thrombin to values comparable with wild type (i.e. ~10$^7$ M$^{-1}$s$^{-1}$) (Fig. 4). However, unlike the effects of pentasaccharide activation, the full-length bridging heparin augmented the reactivities of all chimeras with factors Xa and IXa including the strand 3C chimera beyond that of the pentasaccharide to extents comparable with that of the wild-type inhibitor (i.e. 6–20-fold for factor Xa in the absence of calcium and 100–400-fold for factor IXa in the presence of calcium).

Heparin produced no significant changes in the stoichiometry of inhibition of any of the proteases by the antithrombin chimeras from that of wild-type inhibitor, indicating that the observed changes in the association rate constants for the chimera-protease reactions from wild-type were not due to an altered partitioning of the reaction between inhibition and substrate pathways (13). Together, these results suggested that an important determinant of the pentasaccharide-en-
enhanced reactivity of antithrombin with factors Xa and IXa resides in \(\beta\)-strand 3C of the serpin.

**DISCUSSION**

The purpose of this study was to identify putative exosites in antithrombin that become competent following heparin activation to interact with factors Xa and IXa and thereby enhance the serpin’s reactivity with these target proteases (5, 22, 25). To accomplish this goal, we created six antithrombin chimeras in which residues circumscribing the reactive loop were replaced with the homologous residues of \(\alpha_1\) PI. All of the chimeras behaved like wild-type antithrombin in terms of their ability to bind a specific heparin pentasaccharide with high affinity, undergo heparin pentasaccharide-induced fluorescence changes indicative of conformational activation, and form SDS-stable inhibited complexes with coagulation proteases at a rate that was greatly accelerated by pentasaccharide-containing heparins. Such observations indicate that the \(\alpha_1\) PI replacements in antithrombin do not significantly affect the folding of the inhibitor or the unique ability of this serpin to inhibit coagulation proteases in a manner that is accelerated by sequence-specific heparin binding. Because conserved hydrophobic core residues critical for serpin structure (28) were not altered in the chimeras, the replacements mostly occurred in surface residues (Table I). The chimeras thus afforded an opportunity to evaluate the importance of antithrombin surface residues surrounding the reactive loop in the interactions of the serpin with its major target proteases, thrombin, factor Xa, and factor IXa, and to test recent proposed models for these interactions (43).

Surface residues in strand 3 of \(\beta\)-sheet C were found to be critical for enhancing the reactivity of antithrombin toward both factor Xa and factor IXa following conformational activation of the serpin by heparin pentasaccharide and to therefore contain exosite determinants for factor Xa and factor IXa interaction. Replacement of the antithrombin strand 3C sequence with the homologous sequence from \(\alpha_1\) PI thus abolished most or all (>98%) of the heparin pentasaccharide enhancement in antithrombin reactivity with factors Xa and IXa. By contrast, replacements of other regions surrounding the reactive loop with \(\alpha_1\) PI residues had only small effects on the pentasaccharide enhancement in chimeric reactivity with these proteases. The antithrombin exosites that mediate the reactivity enhancement with factors Xa and IXa thus appear largely intact in all but the strand 3C chimera.

A critical test that the strand 3C substitution had specifically knocked out interactions of heparin-activated antithrombin with factors Xa and IXa was the finding that the strand 3C chimera as well as all other chimeras displayed essentially a normal reactivity with thrombin following pentasaccharide activation. This was expected, given that conformational activation of antithrombin by the pentasaccharide minimally affects its reactivity with thrombin, therefore implying that an exosite does not exist to promote the reaction of activated antithrombin with thrombin (13, 25). Moreover, all antithrombin chimeras including the strand 3C chimera displayed nearly normal full-length heparin-bridging enhancements of their reactivity with the three proteases examined. This bridging enhancement results from the full-length heparin strengthening the protease-antithrombin interaction by providing a site for the protease to bind alongside antithrombin in a ternary complex and was therefore expected to be unaffected by the \(\alpha_1\) PI substitutions (5, 18, 19).

Surprisingly, the reactivity of several of the chimeras with all three proteases differed markedly from that of the wild-type inhibitor in the absence of heparin, despite their normal reactivity with thrombin in the presence of heparin or normal heparin enhancement of their reactivity with factors Xa and IXa. These effects may arise from the substantial changes in the electrostatic charge of antithrombin surface residues produced by the \(\alpha_1\) PI substitutions (Table I) together with the close proximity of the reactive loop to the surface in the native antithrombin conformation (22, 23). The proximity is evident from the ability of the P1 Arg residue of the loop to interact with the serpin body only in the native antithrombin conformation as judged from anisotropy and time-resolved fluorescence changes of a fluorophore-labeled P1 derivative of antithrombin upon pentasaccharide activation (23) as well as from changes in the reactivity of the P1 Arg residue with an arginine deiminase following activation (44). The differential effects of serpin surface charge on the reactivity of native and activated antithrombin chimeras appear to be most dramatic in the case of the reactions with thrombin and less pronounced for the reactions with factors Xa and IXa. However, this is not surprising, given that factors Xa and IXa must interact with an exosite in strand 3C in the activated state and therefore may be involved in more intimate contacts with the serpin surface in the activated state than thrombin. Recent modeling of Michaelis complexes of antithrombin with thrombin and factor Xa support such predicted differences in the contact interfaces of the two proteases with the serpin (43).

Interestingly, the strand 3C chimera, which contains the exosite that interacts with factors Xa and IXa, showed a 20-fold enhanced basal reactivity with thrombin and an enhanced heparin affinity relative to wild-type antithrombin. Strand 3C contains Glu-255, which interacts with the P1 arginine residue of the reactive loop in the antithrombin crystal structure (22, 23). Mutation of Glu-255 to Ala was previously shown to produce a 5-fold stimulation of the basal reactivity of antithrombin with thrombin but not to affect the heparin-activated reactivity (24). Moreover, the mutation produced a 5-fold increase in heparin affinity, similar to the effects of P1 arginine mutations to Trp, His, Leu, or Met (22). The strand 3C chimera effects can thus largely be explained by the breaking of the P1 Arg-Glu 255 interaction and consequent partial activation of the serpin to the high heparin affinity state. Since the E255A antithrombin
The variant was found to have a normal basal reactivity with factor Xa in the previous study (24), the enhanced thrombin basal reactivity of the variant cannot arise from an increased accessibility of the P1 Arg to the protease. Glu-255 must instead specifically hinder the interaction of thrombin with the P1 Arg residue of native antithrombin without affecting the interaction of factor Xa. Modeling of the inhibitor-protease interaction using the heparin cofactor II-S195A thrombin complex structure as a template (45) suggests that this might involve an unfavorable electrostatic interaction of antithrombin Glu-255 with thrombin Glu-192, which would not occur with Gln-192 in factors Xa and IXa. This repulsive interaction may be important for down-regulating antithrombin reactivity with thrombin until antithrombin binds to physiologic heparins.

The changes made in strand 3C include three residues that are highly conserved in vertebrate antithrombins but not in other serpins (46), one of which is Glu-255. Since the Glu-255→Ala mutation minimally affected the pentasaccharide enhancement of antithrombin’s reactivity with factor Xa, this residue cannot be a major contributor to the strand 3C exosite identified to interact with factor Xa in the present study. The other two conserved residues in strand 3C are Tyr-253 and Lys-257. Comparison of native and heparin-activated conformations of antithrombin did not reveal any significant changes in the conformation of these conserved residues in the most refined structures available (47). However, since the structure of the reactive loop is likely to be the most uncertain due to antithrombin crystallizing as a dimer with the reactive loop at the dimer interface, defining the structural changes produced by activation in the vicinity of the reactive loop will require crystallization of antithrombin in a monomer state. It is also possible that structural changes in the exosite residues may not be required to allow interaction of conformationally activated antithrombin with factors Xa and IXa, since the expulsion of the reactive loop from sheet A that accompanies activation may be sufficient to orient the reactive loop and the exosite in a manner that allows simultaneous interactions with these two regions to occur in the activated state but not the native state.

Our localization of factor Xa and factor IXa exosites in strand 3C of antithrombin does not agree with recent proposals that factor Xa exosites exist in strand 4C and in strands 1B and 3B, based on modeling of the antithrombin-factor Xa Michaelis complex (43). However, the modeling study also suggested that thrombin and factor Xa interacted very differently with the antithrombin reactive loop due to the different active site architectures of the proteases and greater length and flexibility of the serpin reactive loop. Given the potential for such different modes of interaction and the absence of a crystal structure of an antithrombin-protease Michaelis complex, the identification of the true exosites in antithrombin can only be revealed through the kind of mutagenesis approach carried out in the present study. Our modeling of the initial noncovalent interaction of factors Xa and IXa with the antithrombin reactive loop, using the heparin cofactor II-S195A thrombin complex as a template, showed that the conserved Tyr-253 residue approaches the 140 loop of factors Xa and IXa. This loop was the three proteases, thrombin, factor Xa, or factor IXa, are shown as separate bar graphs for each protease. The wild-type and chimeric antithrombins are indicated on the left of each graph; reactions of free antithrombin are indicated by black bars, those of antithrombin-heparin pentasaccharide complexes are depicted by gray bars, and those of antithrombin-full-length heparin complexes are shown as striped bars. Rate constants were measured under pseudo-first-order conditions by monitoring the decrease in protease activity as a function of time or heparin concentration as detailed under “Experimental Procedures.” The error bars represent the range or S.E. of at least two measurements.
recently shown to contain a critical conserved Arg residue in both proteases important for interaction with pentasaccharide-activated antithrombin (48, 49). Gln-61 of factor Xa has been also suggested to interact with the exosite in conformationally activated antithrombin (50), yet this residue does not appear to be close enough to strand 3C residues to permit any interaction.

In conclusion, our results establish that strand 3 of β-sheet C of antithrombin contains one or more interaction exosites responsible for enhancing the reactivity of the serpin with factor Xa and factor IXa when conformationally activated by heparin. The exosites enhance antithrombin reactivity with the proteases presumably by providing additional interaction sites for stabilizing the initial Michaelis or subsequent acyl-intermediateserpin-protease complexes (8). Precedence for the utilization of such exosites by serpins in the recognition of their target proteases has come from studies of the serpins, kallistatin and kallistatin-related proteins. The role that particular residues within the strand 3C region of proteases has come from studies of the serpins, kallistatin and Antithrombin exosite

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