Mechanism by Which Exosites Promote the Inhibition of Blood Coagulation Proteases by Heparin-activated Antithrombin*

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Heparin activates the serpin, antithrombin, to inhibit its target blood-clotting proteases by generating new protease interaction exosites. To resolve the effects of these exosites on the initial Michaelis docking step and the subsequent acylation and conformational change steps of antithrombin-protease reactions, we compared the reactions of catalytically inactive S195A and active proteases with site-specific fluorophore-labeled antithrombins that allow monitoring of these reaction steps. Heparin bound to N,N’-dimethyl-N-(acetyl)-N’-(7-nitrobenz-3-oxa-1,3-diazol-4-yl)ethylenediamine (NBD)-fluorophore-labeled antithrombins and accelerated the reactions of the labeled inhibitor with thrombin and factor Xa similar to wild type. Equilibrium binding of NBD-labeled antithrombins to S195A proteases showed that exosites generated by conformationally activating antithrombin with a heparin pentasaccharide enhanced the affinity of the serpin for S195A factor Xa minimally 100-fold. Moreover, additional bridging exosites provided by a hexadecasaccharide heparin activator enhanced antithrombin affinity for both S195A factor Xa and thrombin at least 1000-fold. Rapid kinetic studies showed that these exosite-mediated enhancements in Michaelis complex affinity resulted from increases in $k_{on}$ and decreases in $k_{off}$ and caused antithrombin-protease reactions to become diffusion-controlled. Competitive binding and kinetic studies with exosite mutant antithrombins showed that Tyr-253 was a critical mediator of exosite interactions with S195A factor Xa; that Glu-255, Glu-237, and Arg-399 made more modest contributions to these interactions; and that exosite interactions reduced $k_{off}$ for the Michaelis complex interaction. Together these results show that exosites generated by heparin activation of antithrombin function both to promote the formation of an initial antithrombin-protease Michaelis complex and to favor the subsequent acylation of this complex.

The serpin family protein protease inhibitor, antithrombin, together with its glycosaminoglycan activator, heparin, are the principal anticoagulant regulators of blood coagulation cascade proteases (1, 2). Antithrombin inhibits its target coagulation proteases like other serpins by trapping the enzymes in stable acyl-intermediate complexes (3, 4). These reactions are slow in the absence of heparin but are accelerated several thousandfold by the polysaccharide activator to reach a physiologically significant diffusion-limited rate (5). The acceleration results from heparin binding to antithrombin through a sequence-specific pentasaccharide site and inducing an activating conformational change in the serpin (6–8). Additional saccharides neighboring the pentasaccharide also provide a bridging site for binding the protease and promoting its interaction with antithrombin in a ternary heparin-antithrombin-protease complex (9–13).

Antithrombin like other serpins binds its target proteases through a reactive center loop that contains a substrate recognition sequence for target proteases (1, 2). The x-ray structures of free and heparin pentasaccharide-complexed antithrombin have shown that this reactive loop is partially buried in β-sheet A in the unactivated serpin and is expelled from the sheet and thereby made more accessible to an attacking protease when heparin binds and conformationally activates the protein (14, 15). It was initially thought that this increased accessibility of the serpin reactive loop to protease in the heparin-complexed serpin was the principal basis for the activating effect of heparin (14, 16, 17). However, mutagenesis studies subsequently demonstrated that the ability of the protease to recognize and bind the reactive loop was minimally dependent on the expulsion of the reactive loop from sheet A. Rather the main determinants of the heparin-enhanced reactivity of antithrombin with its target proteases were shown to reside in exosites outside the loop (18–20). These exosites were present both on antithrombin to enhance recognition of factors Xa and IXa (18–20) and on full-length heparin chains to promote the binding of target proteases next to bound antithrombin in a ternary bridging complex (9–13, 20–23).

We recently mapped the exosites on heparin-activated antithrombin that specifically interact with factor Xa and factor IXa by mutagenesis studies (24, 25). Two residues on strand 3 of sheet C, Tyr-253 and Glu-255, which are highly conserved in all vertebrate antithrombins (26), were implicated as exosites that promote antithrombin reactions with factor Xa and factor IXa. More recently, a structure of the ternary heparin pentasaccharide-antithrombin-S195A factor Xa Michaelis complex was reported that has confirmed the importance of these exosite residues and suggested other exosite residues that make contact with factor Xa in the complex (27). Moreover this structure confirmed that the factor Xa autolysis loop constitutes the complementary exosite on the enzyme that interacts with the antithrombin exosite in agreement with prior factor Xa mutagenesis studies (28, 29). Two structures of ternary hepa-

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Antithrombins with mutations of the P72 Ala and P2 residues numbered P1, P2, . . . on the amino-terminal side of the scissile bond and P1′, P2′, . . . on the carboxyl-terminal side of the scissile bond; NBD, N,N′-dimethyl-N-(acetyl)-N-[(7-nitrobenz-3-oxa-1,3-diazol-4-yl)ethyl]enediamine; AMC, 7-amido-4-methylcoumarin; DAPA, dansylarginine piperidinamide; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Boc, t-butoxycarbonyl.

EXPERIMENTAL PROCEDURES

Proteins—Antithrombin was purified from human plasma as described previously (30). Wild-type and variant recombinant antithrombins with an N135Q mutation to eliminate glycosylation heterogeneity at the Asn-135 glycosylation site (31) were expressed in baby hamster kidney cells or in baculovirus-infected insect cells and purified as in previous studies (18, 24, 25). Variant antithrombins with mutations of the P72 Ala and P2′ Leu residues to Cys or in which candidate exosite residues were mutated, were engineered, as in past studies (18, 25). All antithrombins were judged pure by SDS-PAGE analysis. Concentrations were obtained from the 280 nm absorbance using an extinction coefficient of 37,700 M⁻¹ cm⁻¹ (32). Human neutrophil elastase was purchased from Athens Research Technology (Athens, GA), Factor Xa was purchased from Enzyme Research Laboratories (South Bend, IN), and thrombin was obtained from purified prothrombin by activation of the zymogen followed by purification of the active enzyme as described previously (33, 34). Concentrations of active enzyme (typically >90% active) were obtained by measurements of turnover numbers in standard substrate assays that had been calibrated with active site titrated protease (19). S195A thrombin and γ-carboxyglutamic acid-domainless S195A factor Xa were recombinant enzymes expressed in mammalian cells and purified as described previously (35, 36). Concentrations of the catalytically inactive proteases were determined from the 280 nm absorbance using published extinction coefficients (35, 36).

Heparins—Synthetic pentasaccharide and hexadecasaccharide heparins containing the antithrombin binding sequence (5, 37) were provided by Maurice Petitou of Sanofi-Aventis Recherche (Toulouse, France). A full-length heparin containing the pentasaccharide binding sequence and ~50 saccharides in length was isolated from commercial heparin by size and affinity fractionation as described previously (30). Heparin concentrations were based on the concentration of antithrombin binding sites as ascertained by protein fluorescence titrations with the different heparins at protein concentrations well above $K_D$ for the interaction to ensure stoichiometric binding as described previously (7, 30).

Antithrombin Labeling—P7 Cys and P2′ Cys antithrombins were labeled with iodoacetamido-NBD fluorophore by dialyzing ~20 μM protein against labeling buffer (20 mM Hepes, 0.1 M NaCl, pH 8.2) and then incubating for 15 min on ice with a 1.5-fold molar excess of fractionated high affinity heparin and 100 μM dithiothreitol to ensure reduction of the free cysteine. Iodoacetamido-NBD was added to a final concentration of 400 μM, and the mixture was incubated on ice for ~20 h. Unreacted label was removed from the protein, and the protein was buffer-exchanged by chromatography on a PD-10 column equilibrated in 20 mM sodium phosphate, 20 mM NaCl, 1 mM EDTA, pH 7.4 buffer. Heparin was removed from the labeled antithrombin by chromatography on a Mono Q as in the purification of recombinant protein (25), and the protein was concentrated and buffer-exchanged into 50 mM Hepes, 0.1 M NaCl, pH 7.4 by ultrafiltration. The extent of labeling was quantitated from the absorbance of the NBD chromophore at 480 nm using an extinction coefficient of 25,000 M⁻¹ cm⁻¹, and the concentration of protein was determined from the 280 nm absorbance corrected for the contribution of NBD (38). Analytical chromatography of labeled antithrombins on a Hi-Trap heparin column showed that the protein bound to the column with high affinity and was eluted at a salt concentration similar to that of unlabeled antithrombin.

Experimental Conditions—Experiments were done in a physiologic ionic strength (I 0.15) buffer consisting of 100 mM Hepes, 93.5 mM NaCl, 5.0 mM CaCl₂, 0.1% polyethylene glycol 8000, pH 7.4, at 25 °C unless otherwise specified.

Stoichiometries of Antithrombin-Protease Reactions—Thrombin or factor Xa at fixed concentrations ranging from ~50 to 200 nM were incubated with varying concentrations of antithrombin from substoichiometric to a severalfold molar excess over protease in the absence or presence of heparin at levels stoichiometric with antithrombin for a time sufficient to yield >90% completion of the reaction. Residual enzyme activity was then determined by adding 100 μM S2238 for thrombin or 100 μM Spectrozyme FXa for factor Xa, both containing 100 μg/ml Polybrene. Plots of residual enzyme activity versus the molar ratio of inhibitor/enzyme were fit by linear regression, and the stoichiometry was obtained from the abscissa intercept (30).

Association Rate Constants for Antithrombin-Protease Reactions—Antithrombin was mixed with protease in the absence or presence of catalytic levels of heparin with the inhibitor concentration kept minimally at a 5-fold molar excess over
that required to achieve stoichiometric inhibition of the protease. Identical reaction mixtures were quenched at varying times by dilution into substrate, and residual enzyme activity was measured from the initial rate of substrate hydrolysis. Inactivation progress curves were fit by a single exponential function with a zero end point to obtain observed pseudo-first order rate constants ($k_{obs}$). Second order association rate constants were obtained from the slope of plots of $k_{obs}$ versus the antithrombin concentration in the absence of heparin or of $k_{obs}$ versus the heparin concentration when heparin was present. The latter plots required correction for the fraction of added heparin that was bound to antithrombin based on measured $K_D$ values for antithrombin-heparin interactions (30, 31).

**Fluorescence Emission Spectra**—Emission spectra of P7-NBD-antithrombin and P2'-NBD-antithrombin (100–200 nM) in the absence and presence of pentasaccharide or hexadecasaccharide heparins and in the absence or presence of S195A or active thrombin or factor Xa were measured on an SLM 8000 spectrofluorometer from 500 to 650 nm in 5-nm steps. Spectra were repeated at least twice to ensure reproducibility and averaged. Corrections were made for buffer background and dilution. The effects of heparin and protein binding to the labeled antithrombins on the NBD fluorescence were assessed by titrations of these components into the labeled antithrombins monitored by fluorescence changes at the peak wavelength or by addition of levels shown to be saturating followed by recording the spectrum. In cases where incomplete saturation of antithrombin complexes with added ligands was achieved, the spectrum of fully complexed antithrombin was obtained by correcting the spectrum for residual uncomplexed inhibitor using the $K_D$ and maximal fluorescence change determined from independent titrations of the interactions.

**Binding Studies**—Equilibrium dissociation constants for the binding of pentasaccharide or hexadecasaccharide heparins to antithrombin were measured by titrating heparin into solutions of antithrombin at 50–100 nM and monitoring the changes in protein fluorescence emission at 340 nm when excited at 280 nm. Fluorescence changes were fit to the quadratic equilibrium binding equation with $K_D$, the binding stoichiometry, and maximal fluorescence change as the fitted parameters (30). Binding of S195A factor Xa or S195A thrombin to fluorescent labeled antithrombins was done by titrating protease into the labeled antithrombin (100–200 nM) in the absence or presence of pentasaccharide or hexadecasaccharide heparins and monitoring the changes in fluorescence at 540 nm (excitation at 480 nm). Fluorescence changes were fit to the quadratic equilibrium binding equation either assuming a binding stoichiometry of 1 or allowing the stoichiometry to be fit as a parameter along with the $K_D$ and maximal fluorescence change.

Binding of unlabeled wild-type and variant recombinant antithrombins to S195A factor Xa was measured by competitive binding titrations in which the unlabeled antithrombin was titrated into a solution of ternary NBD-antithrombin-heparin-S195A factor Xa complex in the presence of heparin levels sufficient to saturate the added antithrombin. Binding of the unlabeled antithrombin-heparin complex to S195A factor Xa was analyzed by monitoring the decreases in fluorescence due to displacement of the labeled antithrombin-heparin binary complex from the ternary complex with S195A factor Xa. Binding of plasma-derived antithrombin or antithrombin-heparin complexes to S195A thrombin was also studied by competitive binding titrations in which antithrombin was titrated into a solution of thrombin plus the active site fluorescence probe, DAPA, in the absence or presence of pentasaccharide or hexadecasaccharide heparins at levels sufficient to saturate the added antithrombin. Binding of the free or heparin-complexed serpin to S195A thrombin was analyzed from the decrease in fluorescence (excitation at 280 nm, emission at 530 nm) due to displacement of the probe from the thrombin active site. Concentrations of antithrombin-heparin complexes were calculated based on measured $K_D$ values for complex formation (5, 31). Fluorescence changes were fit by the cubic equation for competitive binding to obtain the $K_D$ and the stoichiometry for the binding of antithrombin and antithrombin-heparin complexes to S195A thrombin as well as of antithrombin-heparin complexes to S195A factor Xa (39). Independent analyses of the binding of DAPA to S195A thrombin or thrombin or of P7-NBD-antithrombin-heparin complex to S195A factor Xa were done to fix the $K_D$ and stoichiometry for these interactions.

**Rapid Kinetic Studies**—Rapid kinetic studies of antithrombin-protease reactions or of thrombin-DAPA interactions were done with an Applied Photophysics SX-17MV stopped-flow instrument under pseudo-first order conditions in which the molar concentration of one of the reacting components was maintained at least 5-fold greater than that of the other. For reactions of NBD-labeled antithrombin with proteases, the concentration of protease was varied in molar excess over that of the inhibitor, and reactions were monitored from NBD fluorescence changes using an excitation wavelength of 480 nm and an emission filter with a 520-nm cut-on wavelength. The kinetics of plasma antithrombin reactions with active thrombin and factor Xa were studied using peptide fluorogenic substrates to report the rate of protease inhibition. For these studies, the inhibitor concentration was varied in molar excess over that of the protease, and protease inhibition was monitored from changes in 7-amido-4-methylcoumarin (AMC) fluorescence due to hydrolysis of 5 μM tosyl-GPR-AMC in reactions with thrombin and of 25–50 μM Boc-IEGR-AMC in reactions with factor Xa using an excitation wavelength of 380 nm and an emission filter with a 420 nm cut-on. In most cases, NBD fluorescence changes were fit by a single exponential approach to a final end point fluorescence, and AMC fluorescence changes were fit by a single exponential approach to a final steady-state rate of fluorescence change (40) to obtain the observed pseudo-first order rate constant ($k_{obs}$). Values from at least five reaction traces were averaged. The dependence of $K_{obs}$ on the concentration of the component varied in molar excess was fit by a linear function to obtain $k_{on}$ and $k_{off}$ for one-step binding interactions or by a rectangular hyperbolic function to obtain $K_m$ and $k_{on}$ for the minimal two-step reactions of antithrombin with active proteases as in past studies (38). Corrections for the competitive effect of reporter substrates were made for the thrombin substrate but not for the factor Xa substrate due to the $K_m$ for the latter being >5-fold higher than the substrate concentrations used.
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For reactions of P7-NBD-antithrombin-hexadecasaccharide heparin complexes with thrombin and factor Xa, fluorescence changes were distinctly biphasic and were best fit by a double exponential function. In this case, the dependence of the predominant fast phase \( k_{\text{obs}} \) on protease concentration was analyzed by the hyperbolic function. The slow phase \( k_{\text{obs}} \) showed no significant dependence on protease concentration for either protease (0.2–0.3 s\(^{-1}\)) and was attributed to a first order dissociation of the longer chain heparin from the complex because this phase was not observed for reactions in the absence of heparin or in the presence of the pentasaccharide.

The kinetics of DAPA binding to S195A thrombin or to active thrombin were analyzed by mixing DAPA in molar excess with thrombin and monitoring the increase in fluorescence due to DAPA binding to the enzyme. DAPA fluorescence changes were detected by exciting at 280 nm and observing the emitted fluorescence with a 500-nm cut-on filter. The dependence of \( k_{\text{obs}} \) for DAPA-thrombin interactions on the DAPA concentration was fit by linear regression analysis to obtain \( k_{\text{on}} \) and \( k_{\text{off}} \). The kinetics of binding of plasma antithrombin-hexadecasaccharide heparin complex to S195A thrombin were studied by measuring the decrease in fluorescence due to displacement of DAPA from the thrombin active site as antithrombin-heparin complex is bound. Binding was studied under pseudo-first order conditions in which the concentrations of DAPA and antithrombin-heparin complex were in molar excess over S195A thrombin. The dependence of \( k_{\text{obs}} \) on the antithrombin-heparin complex concentration was fit by the equation for the kinetics of competitive ligand binding (41),

\[
k_{\text{obs}} = k_{\text{off,}L} = \frac{k_{\text{on,}L}[L^*]_0(k_{\text{off,}L} - k_{\text{off,}L})}{k_{\text{on,}L}[L^*]_0 + k_{\text{on,}L}[L]_0 + k_{\text{off,}L}} \tag{1}
\]

where \( k_{\text{on,}L} \) and \( k_{\text{off,}L} \) are on- and off-rate constants for the S195A thrombin-DAPA interaction, \( k_{\text{on,}L} \) and \( k_{\text{off,}L} \) are the on- and off-rate constants for the S195A thrombin-antithrombin-hexadecasaccharide complex interaction, and \([L^*]_0\) and \([L]_0\) are the total concentrations of DAPA and antithrombin-heparin complex, respectively. Because the on-rate constant \( k_{\text{on,}L} \) and dissociation constant \( K_{\text{D,}L} \) for the S195A thrombin-antithrombin-hexadecasaccharide interaction were well determined by the data whereas the off-rate constant \( k_{\text{off,}L} \) was not, \( k_{\text{off,}L} \) in the above equation was replaced by the product \( k_{\text{on,}L} \times K_{\text{D,}L} \). Fitting was done by fixing \( k_{\text{on,}L} \) and \( K_{\text{D,}L} \) at the values determined in independent experiments and allowing \( k_{\text{off,}L} \) and \( k_{\text{on,}L} \) to be the fitted parameters.

The kinetics of binding of wild-type and exosite mutant antithrombins to S195A factor Xa were similarly studied by competitive binding kinetic analyses. In this case unlabeled antithrombin-heparin binary complexes were mixed with ternary heparin-P7-NBD-antithrombin-S195A factor Xa complexes, and the decrease in NBD fluorescence due to displacement of the labeled antithrombin-heparin binary complex from the ternary complex by the unlabeled antithrombin-heparin binary complex was monitored. Molar concentrations of labeled and unlabeled antithrombin-heparin binary complexes were in sufficient excess over S195A factor Xa to cause minimal (<20%) changes in the unbound antithrombin-heparin binary complex concentrations and ensure pseudo-first order conditions. \( k_{\text{obs}} \) was fit by the equation given above for the kinetics of competitive ligand binding. In this case \( k_{\text{on,}L} \) and \( k_{\text{off,}L} \) represent on- and off-rate constants for the binding of NBD-labeled antithrombin-heparin complex to S195A factor Xa, whereas \( k_{\text{on,}L} \) and \( k_{\text{off,}L} \) represent on- and off-rate constants for the binding of unlabeled antithrombin-heparin complex to S195A factor Xa. \( k_{\text{on,}L} \) was fit at the value determined independently from the kinetics of binding of the labeled antithrombin to S195A factor Xa, whereas \( k_{\text{off,}L} \) was fixed at the concentration-independent value of \( k_{\text{obs}} \) measured for displacement of labeled wild-type antithrombin from S195A factor Xa by unlabeled wild-type antithrombin. \( k_{\text{on,}L} \) and \( k_{\text{off,}L} \) were the fitted parameters.

RESULTS AND DISCUSSION

Characterization of Fluorophore-labeled Antithrombins—Recombinant antithrombin variants with P2’ and P7 reactive loop residues replaced with cysteine and labeled with NBD fluorophore were prepared to provide fluorescence sensors of the interactions of antithrombin with catalytically inactive and active forms of the target proteases, thrombin and factor Xa. The introduced single cysteines of the antithrombin variants were stoichiometrically labeled with NBD (~0.7–1.3 mol of NBD/mol of protein) under conditions that were mildly reducing and in which heparin was bound to the serpin to protect the disulfide bonds (42). The labeled antithrombins retained a wild-type affinity for heparin as was evident from the elution of the labeled variants from heparin-agarose at a salt concentration similar to that of the wild-type protein and from titrations, monitored by tryptophan fluorescence changes, of the labeled antithrombins with the high affinity heparin pentasaccharide (not shown). The labeled antithrombins inhibited thrombin and factor Xa with stochiometries greater than the wild-type protein both in the absence and presence of heparin, indicative of an increased flux through the substrate pathway (2); this was most prominent for the P7-labeled inhibitor reactions (Table 1). Nevertheless second order association rate constants for the reactions of the NBD-antithrombins with thrombin and factor Xa through the inhibitory pathway, obtained by multiplying observed association rate constants by the inhibition stochiometries, were comparable to those of the wild-type inhibitor in the case of the P7-labeled serpin and only modestly reduced for the P2’-labeled protein (Table 1).

Perturbations of NBD-antithrombin Fluorescence by Proteases—The fluorescence emission spectra of the NBD-labeled antithrombins showed significant perturbations accompanying the addition of inactive S195A or active wild-type proteases most evident when the antithrombins were complexed with heparins containing the pentasaccharide activating sequence for the serpin (Fig. 1). Heparin binding to the antithrombins itself produced a saturable quenching of ~15% of the NBD fluorescence of P7-NBD-antithrombin and a ~15% enhancement of the fluorescence of P2’-NBD-antithrombin. The addition of S195A factor Xa to heparin- complexed P7- and P2’-NBD-antithrombins caused saturable increases in NBD fluorescence, indicative of Michaelis complex formation. The magnitudes of these increases were ~40% for the P7 label with no shift in the wavelength maximum and ~700% for the P2’
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Addition of active thrombin or factor Xa to the NBD-labeled antithrombins was observed when the reactive loops of the labeled antithrombins were cleaved by neutrophil elastase (44), consistent with the fluorophores reporting the serpin conformational change triggered by protease cleavage in the reactive loop (45). These fluorescence changes resemble those observed previously for other serpins labeled with NBD in their reactive loops when they form covalent complexes with target proteases. The marked P7-NBD fluorescence increase thus arises from burial of the fluorophore beneath helix F when the reactive loop inserts into sheet A and the smaller P2’-NBD quench arises from the increased mobility of the solvent-exposed label with a ∼10-nm blue shift of the peak wavelength. S195A thrombin similarly produced saturable decreases in the NBD fluorescence of the labeled antithrombins complexed with heparin, reflecting Michaelis complex formation. These decreases were ∼2% for the P7 label with no apparent spectral shift and ∼40% for the P2’ label with a ∼10-nm blue shift of the spectrum. The larger changes in intensity and peak wavelength of P2’ than of P7-NBD fluorescence spectra produced by the binding of the labeled antithrombins to S195A proteases are in keeping with the P2’ residue being in close contact with the protease active site but the P7 residue lying outside of the contact region with protease. The observed enhancements of NBD fluorescence upon binding S195A factor Xa but quenching of fluorescence on binding S195A thrombin additionally underscore the marked differences seen in the x-ray structures of the two antithrombin-protease Michaelis complexes (10, 11, 27) with factor Xa making a more intimate exosite-mediated interaction with the antithrombin reactive loop than thrombin in the complexes.

Addition of active thrombin or factor Xa to the NBD-labeled antithrombins produced changes in NBD fluorescence completely different from the inactive proteases in keeping with the major conformational change that accompanies the conversion of the noncovalent Michaelis complex to a covalent acyl-intermediate complex (3, 43). Both thrombin and factor Xa produced similar large 280% enhancements and ∼10-nm blue shifts in NBD fluorescence of the P7-labeled antithrombin, whereas a modest ∼20% quenching of the fluorescence of P2’-NBD-antithrombin was observed upon reaction with either of the two proteases. Fluorescence changes similar to those produced by reaction of the labeled antithrombins with active proteases were observed when the reactive loops of the labeled antithrombins were cleaved by neutrophil elastase (44), consistent with the fluorophores reporting the serpin conformational change triggered by protease cleavage in the reactive loop (45). These fluorescence changes resemble those observed previously for other serpins labeled with NBD in their reactive loops when they form covalent complexes with target proteases. The marked P7-NBD fluorescence increase thus arises from burial of the fluorophore beneath helix F when the reactive loop inserts into sheet A and the smaller P2’-NBD quench arises from the increased mobility of the solvent-exposed

### Table 1

| Reaction with thrombin | Antithrombin | No heparin | +H5 | +H16 |
|------------------------|--------------|------------|-----|------|
| **N135Q**              |              |            |     |      |
| $k_a$ (s^{-1})         | 5.8 ± 0.1 × 10^{12} | 7.0 ± 0.7 × 10^{12} | 4.4 ± 1.9 × 10^{12} | 7 ± 3 × 10^{10} |
| $SI$ (mol AT/mol T)    | 1.0 ± 0.1 × 10^{4} | 1.2 ± 0.2 × 10^{4} | 3.3 ± 1.8 × 10^{4} | 1.0 ± 0.6 × 10^{4} |
| $k_a/SI$               | 7.6 ± 0.7 × 10^{10} | 4.2 ± 0.3 × 10^{10} | 11 ± 1.5 × 10^{10} | 9 ± 3 × 10^{8} |

| Reaction with factor Xa | Antithrombin | No heparin | +H5 | +H16 |
|-------------------------|--------------|------------|-----|------|
| **N135Q**               |              |            |     |      |
| $k_a$ (s^{-1})         | 4.3 ± 0.3 × 10^{12} | 1.5 ± 0.3 × 10^{12} | 1.5 ± 0.5 × 10^{12} | 9.0 ± 0.6 × 10^{12} |
| $SI$ (mol AT/mol Xa)    | 1.1 ± 0.1 × 10^{4} | 1.3 ± 0.3 × 10^{4} | 1.4 ± 0.5 × 10^{4} | 1.4 ± 0.5 × 10^{4} |
| $k_a/SI$               | 5.7 ± 0.4 × 10^{10} | 5.7 ± 0.4 × 10^{10} | 6.6 ± 0.4 × 10^{10} | 6.6 ± 0.4 × 10^{10} |

Values were measured at 25°C in 0.15, pH 7.4 Hepes buffer plus calcium ions from discontinuous assays of the rate of antithrombin inhibition of proteases or from stoichiometric end point titrations of antithrombin-protease reactions as described under "Experimental Procedures." H5, pentasaccharide heparin; H16, hexadecasaccharide heparin; Xa, factor Xa; T, thrombin; AT, antithrombin.

Label with a ∼10-nm blue shift of the peak wavelength. S195A thrombin similarly produced saturable decreases in the NBD fluorescence of the labeled antithrombins complexed with heparin, reflecting Michaelis complex formation. These decreases were ∼2% for the P7 label with no apparent spectral shift and ∼40% for the P2’ label with a ∼10-nm blue shift of the spectrum. The larger changes in intensity and peak wavelength of P2’- than of P7-NBD fluorescence spectra produced by the binding of the labeled antithrombins to S195A proteases are in keeping with the P2’ residue being in close contact with the protease active site but the P7 residue lying outside of the contact region with protease. The observed enhancements of NBD fluorescence upon binding S195A factor Xa but quenching of fluorescence on binding S195A thrombin additionally underscore the marked differences seen in the x-ray structures of the two antithrombin-protease Michaelis complexes (10, 11, 27) with factor Xa making a more intimate exosite-mediated interaction with the antithrombin reactive loop than thrombin in the complexes.
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Binding of S195A Factor Xa to the Labeled Antithrombins—
The ~40% fluorescence increase accompanying the addition of S195A factor Xa to P7-NBD-antithrombin-heparin complex allowed quantitation of the binding affinity of the inactive factor Xa for antithrombin in the absence and presence of the heparin pentasaccharide and a hexadecasaccharide heparin mimic capable of bridging antithrombin and proteases (37). Titrations of S195A factor Xa into P7-NBD-antithrombin complexed with the natural pentasaccharide, a higher affinity pentasaccharide, (5) or the hexadecasaccharide heparin resulted in saturable increases in fluorescence of comparable magnitude (Fig. 2A). However, saturation was achieved at lower protease concentrations in the case of the hexadecasaccharide heparin-complexed antithrombin than with the pentasaccharide-complexed inhibitor. Fitting of the data by the equilibrium binding equation yielded $K_D$ values of $121 \pm 19$, $125 \pm 21$, and $8.7 \pm 1.7$ nM for natural pentasaccharide-, high affinity pentasaccharide-, and hexadecasaccharide-complexed inhibitors, respectively (average values ± S.E. from four to five titrations for each heparin). A minor fluorescence increase was detected when the labeled antithrombin was titrated with S195A factor Xa in the absence of heparin over the same range of protease concentration, implying a very weak binding interaction ($K_D \geq 30 \mu M$).

Activation of antithrombin by pentasaccharide and hexadecasaccharide heparins thus results in minimal enhancements of ~100- and ~1000-fold, respectively, in affinity for S195A factor Xa that fully account for the rate-enhancing effects of these heparins on antithrombin-factor Xa reactions.

Titrations of P2'-NBD-antithrombin with S195A factor Xa similarly showed that complexation of the labeled serpin with pentasaccharide and hexadecasaccharide heparins resulted in a saturable binding of S195A factor Xa to form a Michaelis complex. However, the $K_D$ values of 6000 ± 2800 and 1300 ± 300 nM for these interactions were considerably weaker (~100-fold) than those for the P7-labeled inhibitor interactions (Fig. 2B).

P7- and P2'-labeled antithrombin-heparin binary complexes could be competitively displaced from the ternary complexes they formed with S195A factor Xa by unlabeled wild-type antithrombin-heparin binary complex. This was evident from the ability to completely reverse the fluorescence increases by titrating the unlabeled antithrombin into the labeled antithrombin-S195A factor Xa complexes in the presence of saturating heparin (not shown). Analysis of such competitive binding titrations yielded indistinguishable $K_D$ values for the unlabeled antithrombin-S195A factor Xa interactions that were similar to the $K_D$ obtained for the P7-labeled inhibitor interaction but ~100-fold lower than the $K_D$ measured for the P2'-labeled inhibitor interaction (see Fig. 8 below and Table 4). The P7 label is thus nonperturbing, whereas the P2' label perturbs the antithrombin-protease interaction. This is consistent with the latter label lying within the reactive loop region that contacts the protease active site and with the observed effects of the P2' label on $k_{cat}$ (Table 1) (38).

Binding of S195A Thrombin to the Labeled Antithrombins—
Binding of S195A thrombin to P2'-NBD-antithrombin was barely detectable in the absence of heparin or when the labeled inhibitor was activated by heparin pentasaccharide ($K_D \approx 5 \mu M$). However, a saturable fluorescence quenching was observable in the presence of the hexadecasaccharide heparin that indicated a reasonably high affinity $K_D$ of 24 ± 2 nM (Fig. 3A).

Because of the small change in fluorescence induced by the binding of S195A thrombin to P7-NBD-antithrombin-heparin complex (Fig. 1A) and the finding that the P2' label interfered with the binding of S195A factor Xa, binding of S195A thrombin to antithrombin was further studied using the active site-directed fluorescence probe DAPA (46). Binding was analyzed by titrating plasma antithrombin into a solution of the S195A thrombin-DAPA complex with or without heparin present and monitoring the decrease in DAPA fluorescence due to displacement of the probe from the active site of thrombin as antithrombin is bound. Minimal displacement of DAPA from S195A thrombin resulted from titrating in free antithrombin or...
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FIGURE 3. Fluorescence titrations of antithrombin-S195A thrombin interactions. A, titrations of 200 nM P2'-NBD-antithrombin in the absence of heparin (AT, ●) or in the presence of 450 nM pentasaccharide (AT-H5, ▲) or 400 nM hexadecasaccharide (AT-H16, ■) heparins monitored by NBD fluorescence changes. Solid lines are fits to the quadratic equilibrium binding equation. B, titrations of 200 nM S195A thrombin and 300 nM DAPA with antithrombin alone (●), antithrombin-pentasaccharide complex (▲), or antithrombin-hexadecasaccharide complex (■) monitored from decreases in DAPA fluorescence due to displacement of DAPA from the enzyme active site upon inhibitor binding. Additional titrations of 200 nM S195A thrombin and 200 nM DAPA (▲ or □) or 300 nM S195A thrombin and 300 nM DAPA (■) with antithrombin-hexadecasaccharide complex are shown. Heparin concentrations were fixed at 1–1.5 μM, and concentrations of antithrombin-heparin complexes (AT-H) were calculated from measured K_D values. Solid lines are fits to the competitive binding equation with the K_D and stoichiometry for the S195A thrombin-DAPA interaction fixed at average values determined from three independent equilibrium binding titrations. F, fluorescence.

FIGURE 4. Kinetics of S195A factor Xa binding to P7-NBD-antithrombin-heparin complexes. P7-NBD-antithrombin (25–100 nM) and a 1.2-fold molar excess of either pentasaccharide (AT-H5, ▲) or hexadecasaccharide (AT-H16, ■) heparins were mixed with at least a 5-fold molar excess of S195A factor Xa (FXa) in the stopped-flow fluorometer under pseudo-first order conditions. The exponential increase in NBD fluorescence was then monitored as a function of time and computer fit to obtain k_off as described under “Experimental Procedures.” Average values of k_off are plotted as a function of the S195A factor Xa concentration and fitted by linear regression (solid lines).

the antithrombin-pentasaccharide complex (K_D values >2 μM), whereas full DAPA displacement was observed in titrations with the antithrombin-hexadecasaccharide complex (Fig. 3B). Analysis of the displacement curves at three different concentrations of thrombin-probe complex showed that the data could be fit by an average K_D of 9.3 ± 0.3 nM. It should be noted that our use of the heparin hexadecasaccharide mimetic that contains only a single productive site for binding thrombin (37) avoided nonproductive binding of thrombin to heparin sites not adjacent to bound antithrombin. Such nonproductive binding was evident in previous studies from the inability to completely displace a thrombin active site probe by antithrombin-heparin complex (9, 46). Our results thus show that the hexadecasaccharide bridging heparin produces an enhancement of the antithrombin-S195A thrombin Michaelis complex affinity that approaches the several thousandfold activating effect of this heparin on k_a for antithrombin inhibition of thrombin.

Kinetics of S195A Factor Xa-Antithrombin Interactions—The fluorescence changes used to monitor the equilibrium binding interactions of S195A proteases with antithrombin-heparin complexes were also used to study the kinetics of these interactions using stopped-flow methods. The rate of binding of S195A factor Xa to P7-NBD-antithrombin-heparin complex under pseudo-first order conditions was observable from an exponential increase in fluorescence over the range of protease concentrations tested. The observed pseudo-first order rate constant (k_off) increased linearly with increasing protease concentration for the binding of S195A factor Xa to both pentasaccharide- and hexadecasaccharide-complexed antithrombins, consistent with a single step binding process (Fig. 4). On-rate constants (k_on) obtained from the slope and off-rate constants (k_off) obtained from the intercept of these plots were 9.9 ± 0.1 × 10^5 M^{-1} s^{-1} and 0.07 ± 0.01 s^{-1} for the antithrombin-pentasaccharide complex interaction and 1.2 ± 0.1 × 10^7 M^{-1} s^{-1} and 0.5 ± 0.1 s^{-1} for the antithrombin-hexadecasaccharide complex interaction. The ratio of k_off/k_on showed fair agreement with the measured K_D for the former interaction but not the latter interaction, most likely due to the greater error in k_off. Direct determination of k_off for the latter interaction by competitive binding kinetic studies discussed later gave a value of ~0.2 s^{-1}, yielding a ratio of k_off/k_on in more reasonable agreement with K_D. These results indicate that the heparin pentasaccharide enhancement of the affinity of antithrombin for factor Xa in the Michaelis complex correlates with a low off-rate constant and that the additional enhance-
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ment in affinity produced by the bridging heparin is due to an increase in the on-rate constant.

Kinetics of S195A Thrombin-Antithrombin Interactions—The kinetics of binding of the antithrombin-hexadecasaccharide heparin complex to S195A factor Xa were analyzed under pseudo-first conditions by monitoring the time-dependent decrease in fluorescence due to displacement of DAPA from the thrombin active site as antithrombin-heparin complex is bound. In contrast to the linear dependence of the binding rate constant on the varied component observed for heparin-antithrombin-S195A factor Xa interactions, $k_{\text{obs}}$ for antithrombin-heparin complex binding to S195A thrombin showed a saturable dependence on antithrombin-heparin complex concentration that approached a limiting value of $7 - 8 \text{ s}^{-1}$ (Fig. 5A) in agreement with a previous report (46). Analysis of the kinetics of DAPA binding to S195A thrombin or to active thrombin to form binary complexes showed that $k_{\text{obs}}$ increased linearly with increasing DAPA concentration, consistent with a simple equilibrium binding of the probe to the enzyme active site (Fig. 5B). The slopes and intercepts of these plots indicated similar on-rate constants of $4.2 \pm 0.2 \times 10^7$ and $4.1 \pm 0.1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ but ~20-fold different off-rate constants of $8.6 \pm 0.9$ and $0.4 \pm 0.2 \text{ s}^{-1}$ for DAPA interactions with S195A and active thrombin, respectively. These results were in keeping with the ~20-fold lower affinity of the probe for the inactive enzyme ($K_D$, $160 \pm 5 \text{ nm}$) than for the active enzyme ($K_D$, $12 \pm 2 \text{ nm}$) measured in equilibrium binding titrations and with previous studies showing the perturbing effects of the S195A mutation on the S1 binding pocket (47). Notably the off-rate constant for DAPA binding to S195A thrombin of $8.6 \pm 0.9 \text{ s}^{-1}$ was experimentally indistinguishable from the limiting rate constant of $7.5 \pm 0.1 \text{ s}^{-1}$ for displacement of the probe from S195A thrombin by antithrombin-heparin complex, suggesting that the rate of antithrombin-heparin complex binding to the inactive protease was becoming limited by the slow off-rate of DAPA from the protease at high concentrations of antithrombin-heparin complex. The observed saturation kinetics therefore do not reflect a rate-limiting conformational change in antithrombin induced by thrombin binding as previously concluded (46). The kinetic data are instead consistent with the ternary heparin-antithrombin-thrombin Michaelis complex being a simple docking complex like the ternary heparin-antithrombin-factor Xa Michaelis complex with no major conformational changes induced in the serpin or protease in these complexes in keeping with the x-ray structures of the complexes (10, 11).

Fitting of the data by the equation for the kinetics of displacement of a ligand by a competing ligand (see “Experimental Procedures”) and requiring that $k_{\text{off}}/k_{\text{on}}$ for S195A thrombin binding to the antithrombin-hexadecasaccharide heparin complex be equal to the measured $K_D$ for the interaction allowed fitting of a $k_{\text{off}}$ of $3.6 \pm 0.2 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ for the interaction from which a $k_{\text{on}}$ of $3.3 \text{ s}^{-1}$ was calculated. The on-rate constant for the heparin-antithrombin-thrombin Michaelis complex interaction is thus substantially higher than that for the heparin-antithrombin-factor Xa interaction, suggesting a more favorable electrostatically guided on-rate for thrombin than for factor Xa in forming the docking complex. This may reflect the longer heparin requirement to produce an optimal bridging rate enhancement for the reaction of antithrombin with factor Xa than with thrombin (48). The x-ray structures of the Michaelis complexes indeed show that for factor Xa to engage the antithrombin exosite and the heparin bridging exosite at the same time in the complex a longer chain heparin than is necessary to bind thrombin would be required (10, 11, 27).
Kinetics of Antithrombin Reactions with Active Proteases—
The large increases in fluorescence of P7-NBD-antithrombin produced upon forming the trapped acyl-intermediate complex with active factor Xa or thrombin were used to measure the kinetics of formation of the intermediate Michaelis complex in these reactions. Reactions of factor Xa or thrombin with P7-NBD-antithrombin complexed with the hexadecasaccharide heparin under pseudo-first order conditions resulted in a saturable dependence of $k_{\text{obs}}$ on protease concentration for both protease reactions (Figs. 6A and 7A). From this dependence, a $K_m$ value for formation of the Michaelis complex of $2.6 \pm 0.3 \, \mu M$; a limiting rate constant, $k_{\text{lim}}$, for conversion of the Michaelis complex to the trapped acyl-intermediate complex of $12 \pm 1 \, s^{-1}$; and overall $k_{\text{cat}}$, reflecting the ratio $k_{\text{lim}}/K_m$, of $4.6 \pm 0.9 \times 10^6 \, M^{-1} \, s^{-1}$ were determined for the reaction with factor Xa. Moreover a $K_m$ of $37 \pm 3 \, nM$, $k_{\text{lim}}$ of $1.8 \pm 0.1 \, s^{-1}$, and overall $k_{\text{cat}}$ of $4.9 \pm 0.7 \times 10^6 \, M^{-1} \, s^{-1}$ were obtained for the reaction with thrombin. Reactions of the labeled antithrombin in the absence of heparin or complexed with the heparin pentasaccharide showed no evidence for saturation of the Michaelis complex with either protease. The linear dependence of $k_{\text{obs}}$ on protease concentration for these reactions yielded $k_{\text{obs}}$ values of $7.3 \pm 0.2 \times 10^3$ and $1.2 \pm 0.3 \times 10^4 \, M^{-1} \, s^{-1}$ for free and pentasaccharide-complexed antithrombin reactions with factor Xa and values of $1.0 \pm 0.1 \times 10^4$ and $2.0 \pm 0.1 \times 10^5 \, M^{-1} \, s^{-1}$ for the corresponding reactions with thrombin. The $k_{\text{obs}}$ values measured for the labeled antithrombin reactions under pseudo-first order conditions from changes in NBD fluorescence using a molar excess of protease were in all cases similar to those measured from losses in enzyme activity using a molar excess of labeled serpin only after the latter were corrected for the fraction of the serpin that reacted along the inhibitory pathway (Tables 1 and 3). This was predicted to be the case from kinetic simulations.

To ensure that the kinetic parameters measured for the P7-NBD-antithrombin-protease reactions reflected those of unlabeled antithrombin-protease reactions, the kinetics of plasma antithrombin-protease reactions were measured in the presence of different chain length heparins under pseudo-first order conditions using a reporter fluorogenic substrate to monitor the reactions (Figs. 6B and 7B). Saturation kinetics were observed for all heparin-promoted reactions of antithrombin with factor Xa that indicated decreasing $K_m$ values of $30 \pm 14$, $6.0 \pm 1.1$, and $1.3 \pm 0.2 \, \mu M$ for pentasaccharide, hexadecasaccharide, and $50$-saccharide heparin-accelerated reactions but similar $k_{\text{lim}}$ values of $25–28 \, s^{-1}$ for these reactions (Fig. 6B and Table 3). A saturable increase in $k_{\text{obs}}$ with increasing antithrombin-heparin concentration was also observed for the reaction of plasma antithrombin-hexadecasaccharide heparin complex with thrombin, the data yielding a $K_m$ of $45 \pm 6 \, nM$, $k_{\text{lim}}$ of $4.7 \pm 0.2 \, s^{-1}$, and $k_{\text{lim}}/K_m$ of $1.0 \pm 0.2 \times 10^8 \, M^{-1} \, s^{-1}$ (Fig. 7B and Table 3). Correction of the overall association rate constants, given by the ratio $k_{\text{lim}}/K_m$ for the fraction of serpin reacting through the inhibitory pathway (Table 1) gave $2–4$-fold higher values for plasma antithrombin reactions than for the corresponding P7-NBD-antithrombin reactions, verifying modest effects of the NBD label on the kinetic parameters for the inhibitory reactions. Together these findings indicated that full-length heparins promoted antithrombin-protease reactions by decreasing $K_m$, consistent with the observed effects of heparin-dependent exosites on the affinities of the Michaelis complexes.

Comparison of Exosite Effects on $K_m$ and $K_D$—Comparison of the kinetic data for antithrombin reactions with active proteases with the binding data for antithrombin interactions with S195A proteases notably revealed that the $K_m$ values characterizing the Michaelis complexes with active proteases substan-
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![Diagram](image)

FIGURE 7. Kinetics of P7-NBD-antithrombin and plasma antithrombin reactions with thrombin. A, P7-NBD-antithrombin (4–25 nM) alone (AT, ) or in the presence of a 4–10-fold molar excess of pentasaccharide (AT-H5, □) or hexadecasaccharide (AT-H16, □) heparin was mixed with a molar excess of thrombin (minimally 10 times greater than the concentration of inhibited complex formed in the reaction) in the stopped-flow fluorometer. The exponential increase in NBD fluorescence was then monitored with time to obtain $k_{\text{obs}}$ for the reaction with protease. $k_{\text{obs}}$ values measured as a function of the thrombin concentration were fit by linear regression for antithrombin and antithrombin-pentasaccharide complex reactions (solid lines) and by a hyperbolic function for the antithrombin-hexadecasaccharide complex reaction (inset) and by a hyperbolic function for the antithrombin-hexadecasaccharide complex reaction (inset) and by a hyperbolic function for the antithrombin-pentasaccharide complex reactions (dashed line) for antithrombin and antithrombin-pentasaccharide complex reactions (solid lines). B, thrombin (0.5–6 nM) was mixed with at least a 100-fold molar excess of thrombin plus a limiting concentration of hexadecasaccharide heparin (calculated antithrombin-heparin complex concentration was minimally 10-fold greater than the thrombin concentration) and 5 μM tosyl-GPR-AMC substrate in the stopped-flow fluorometer. The exponential decrease in rate of hydrolysis of the fluorescence substrate was then monitored to obtain $k_{\text{lim}}$. The dependence of $k_{\text{lim}}$ on the concentration of antithrombin-heparin complex corrected for the competitive effect of substrate was fit by a rectangular hyperbolic function (solid line). A measured $K_m$ of 4.5 μM for thrombin hydrolysis of the substrate was used to calculate the correction factor.

> Values were obtained from fits of the data of Figs. 2–5 as described in the legends to these figures and under “Experimental Procedures.” H5, pentasaccharide heparin; H16, hexadecasaccharide heparin.

| Antithrombin | No heparin | +H5 | +H16 |
|--------------|------------|-----|------|
| Reaction with S195A thrombin | | | |
| Plasma | $K_m$ (μM) | $k_{\text{lim}}$ (s$^{-1}$) | $k_{\text{lim}}/K_m$ (μM$^{-1}$ s$^{-1}$) |
| +H5 | $>30$ | 0.121 ± 0.019 | 0.0087 ± 0.0017 |
| +H16 | $>30$ | 0.099 ± 0.01 | 11.5 ± 0.5 |
| P7-NBD | $K_m$ (μM) | $k_{\text{lim}}$ (s$^{-1}$) | $k_{\text{lim}}/K_m$ (μM$^{-1}$ s$^{-1}$) |
| +H5 | $>30$ | 0.07 ± 0.01 | 0.5 ± 0.1 |
| +H16 | $>30$ | 0.024 ± 0.002 | |

**TABLE 3**

Kinetic constants for reactions of P7-NBD antithrombin and plasma antithrombin with thrombin and factor Xa

Values for kinetic parameters were obtained from fits of the data of Figs. 6 and 7 either by a straight line or by a rectangular hyperbolic function as indicated in the figure legends and under “Experimental Procedures.” H5, pentasaccharide heparin; H16, hexadecasaccharide heparin; H50, 50-saccharide heparin.

| Antithrombin | No heparin | +H5 | +H16 |
|--------------|------------|-----|------|
| Reaction with thrombin | | | |
| Plasma | $K_m$ (μM) | $k_{\text{lim}}$ (s$^{-1}$) | $k_{\text{lim}}/K_m$ (μM$^{-1}$ s$^{-1}$) |
| +H5 | $>30$ | 0.045 ± 0.006 | 4.7 ± 0.2 |
| +H16 | $>30$ | 0.0090 ± 0.003 | 0.015 ± 0.001 |
| P7-NBD | $K_m$ (μM) | $k_{\text{lim}}$ (s$^{-1}$) | $k_{\text{lim}}/K_m$ (μM$^{-1}$ s$^{-1}$) |
| +H5 | $>30$ | 0.37 ± 0.003 | 1.8 ± 0.1 |
| +H16 | $>30$ | 0.010 ± 0.001 | 0.020 ± 0.001 |

| Antithrombin | No heparin | +H5 | +H16 |
|--------------|------------|-----|------|
| Reaction with factor Xa | | | |
| Plasma | $K_m$ (μM) | $k_{\text{lim}}$ (s$^{-1}$) | $k_{\text{lim}}/K_m$ (μM$^{-1}$ s$^{-1}$) |
| +H5 | $>30$ | 30 ± 14 | 6.0 ± 1.1 |
| +H16 | $>30$ | 28 ± 10 | 25 ± 3 |
| P7-NBD | $K_m$ (μM) | $k_{\text{lim}}$ (s$^{-1}$) | $k_{\text{lim}}/K_m$ (μM$^{-1}$ s$^{-1}$) |
| +H5 | $>30$ | 2.6 ± 0.3 | |
| +H16 | $>30$ | 12 ± 1 | |

$^a$ Values were taken from a prior study (5).

tially exceeded measured $K_D$ values for Michaelis complexes with S195A proteases by as much as 300-fold (Tables 2 and 3). This difference between $K_P$ and $K_m$ may be even greater because the affinities of Michaelis complexes measured with S195A proteases may be underestimated based on present and past observations that the S195A mutation weakens the S1 binding pocket interaction by 10–20-fold (Fig. 5B) (42). Observed $K_m$ values therefore do not represent equilibrium dissociation constants for the Michaelis complex interactions but rather reflect a steady-state formation of the Michaelis complex during the reaction. This observation implies that the Michaelis complexes undergo acylation before they have a chance to dissociate back to free serpin and protease. Indeed the limiting rate constants measured for the forward acylation/conformational change reactions of the Michaelis complexes with active proteases exceed measured off-rate constants for the Michaelis complexes with S195A proteases; this is most pronounced with factor Xa. The differential between $k_{\text{lim}}$ and $k_{\text{off}}$ is likely to be large for both protease reactions given that the S195A mutation perturbs $k_{\text{off}}$ and not $k_{\text{lim}}$. When $k_{\text{lim}} \gg k_{\text{off}}$, the $K_m$ that is given by the ratio $(k_{\text{off}} + k_{\text{lim}})/k_{\text{lim}}$ approaches $k_{\text{lim}}/k_{\text{off}}$, and the overall association rate constant for the reaction, given by the ratio $k_{\text{lim}}/k_{\text{off}}$, becomes $k_{\text{lim}}/(k_{\text{lim}}/k_{\text{off}}) = K_m$. In such a case the initial encounter of serpin and protease commits the reacting proteins to undergo the subsequent acylation and conformational change steps leading to irreversible protease inhib-
tion, and the reaction becomes limited by the rate of the diffusional encounter of serpin and protease. That the reactions of antithrombin-pentasaccharide complex with factor Xa and of antithrombin-hexadecasaccharide complex with both factor Xa and thrombin nearly achieve this situation is suggested by the finding that second order association rate constants closely approach measured $k_{on}$ values for formation of the Michaelis complexes as predicted (Table 2). Exosites thus result in the serpin-protease reactions operating close to the maximum possible efficiency.

Conversely antithrombin reactions in the absence of exosites are associated with much weaker Michaelis complex affinities and higher off-rate constants, and therefore, $K_m$ is expected to be limited both by the $k_{on}$ and $k_{off}$ for the initial Michaelis complex interaction as well as the $k_{lim}$ for the acylation/conformational change steps beyond the Michaelis complex.

Relative Contributions of Exosite Residues to Promoting the Antithrombin-Factor Xa Michaelis Complex Interaction—We previously mapped the exosite on antithrombin for binding factor Xa and factor IXa to residues Tyr-253 and Glu-255 by mutagenesis studies (24, 25), and its localization was recently confirmed by an x-ray structure of the pentasaccharide-antithrombin-S195A factor Xa complex (27). To quantitate the relative contributions of exosite residues to enhancing the antithrombin-S195A factor Xa Michaelis complex interaction, we performed competitive binding titrations in which the binding of unlabeled mutant antithrombin-heparin binary complexes to S195A factor Xa was measured from the decrease in fluorescence resulting from displacement of P7-NBD-antithrombin-heparin binary complex from its ternary complex with S195A factor Xa (Fig. 8). No binding was detected when Tyr-253 was mutated to Ala or Lys alone or when the Y253A mutant was combined with an E255A mutation, indicating that the mutation of the critical Tyr-253 exosite residue reduced the binding affinity at least 10-fold relative to the wild-type serpin interaction (Table 4). The more conservative change of Tyr-253 to Phe caused a ~3-fold reduction in affinity, suggesting that the phenyl ring was more important than the hydroxyl substituent on the ring in determining the exosite interaction with S195A factor Xa. Mutations of Glu-255 to Leu, Gln, or Arg also resulted in significant reductions of 6–8-fold in antithrombin binding affinity for S195A factor Xa. By contrast, mutation of other residues resulted in more modest or no decreases in affinity compared with the wild-type interaction. Thus mutation of Gln-254 to Arg reduced the affinity ~4-fold, mutation of Glu-237 to Ala caused ~3-fold weaker binding, mutations of Arg-399 to Met or Glu reduced affinity 2–6-fold, and mutations of Lys-257 to Met or Glu had no significant effect on binding. A ~30-fold weaker binding than the wild-type interaction was measured for the Y253K mutant when the hexadecasaccharide heparin was used to activate antithrombin and thereby increase the affinity of the Michaelis complex interaction. However, the Y253A single or Y253A/E255A double mutants complexed with the hexadecasaccharide still were unable to bind over the range of concentrations experimentally feasible (up to ~500 nm, not shown). Tyr-253 thus appears to be the most critical determinant of the heparin-activated antithrombin-S195A factor Xa Michaelis complex interaction.

To determine how antithrombin exosite residues enhance binding to factor Xa, competitive binding kinetic studies were performed in which we compared the rate at which unlabeled wild-type and mutant antithrombin-heparin binary complexes displaced P7-NBD-antithrombin-heparin binary complex from
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a ternary complex with S195A factor Xa. Unlabeled wild-type antithrombin displaced the labeled antithrombin with a rate constant of $0.216 \pm 0.004 \text{ s}^{-1}$ that was independent of unlabeled inhibitor concentration (Fig. 9). This result was consistent with equivalent $k_{on}$ and $k_{off}$ values of $12 \text{ M}^{-1} \text{s}^{-1}$ and 0.2 s$^{-1}$ for the interaction of labeled and unlabeled antithrombin-heparin complexes with S195A factor Xa. By contrast, Y253F and E237A mutant antithrombins displaced P7-NBD-antithrombin from S195A factor Xa with faster rate constants that hyperbolically approached the $k_{off}$ for the labeled antithrombin as the concentration of the mutant antithrombin was increased. These data yielded $k_{on}$ and $k_{off}$ values of $14 \pm 3 \text{ M}^{-1} \text{s}^{-1}$ and 0.61 $\pm$ 0.05 s$^{-1}$ for the Y253F antithrombin-heparin complex interaction with S195A factor Xa and $17 \pm 2 \text{ M}^{-1} \text{s}^{-1}$ and 1.4 $\pm$ 0.1 s$^{-1}$ for the analogous E237A antithrombin ternary complex interaction. These results indicate that the weaker affinities of the exosite mutants for S195A factor Xa result from increased values for $k_{off}$. The exosite interaction thus enhances the affinity of the antithrombin-S195A factor Xa Michaelis complex by lowering $k_{off}$.

Interestingly the effects of mutating antithrombin exosite residues on the heparin-antithrombin-factor Xa Michaelis complex interaction do not exactly parallel the effects of the mutations on $k_a$ for the pentasaccharide-accelerated antithrombin-factor Xa reaction that we reported previously (Table 4) (25). In particular, most single residue mutations have small effects on $k_a$ and only single or double mutants that include Tyr-253 produce large effects (25). This can now be rationalized based on our finding that heparin-generated

FIGURE 9. Competitive binding kinetics of unlabeled and NBD-labeled antithrombin-heparin binary complexes to S195A factor Xa. Wild-type (○), Y253F (△), and E237A (×) antithrombins (AT) were mixed in the stopped-flow fluorometer with ternary S195A factor Xa-P7-NBD-antithrombin-heparin hexadecasaccharide (H16) complex formed from 50 nM S195A factor Xa, 125 nM P7-NBD-antithrombin, and a large molar excess of heparin (300–2000 nM) that was sufficient to complex the unlabeled antithrombin. $k_{obs}$ for binding of the unlabeled antithrombin-heparin binary complex to S195A factor Xa was monitored from the exponential decrease in NBD fluorescence accompanying the displacement of the labeled antithrombin-heparin binary complex from the ternary complex under conditions where antithrombin-heparin binary complexes were in molar excess over S195A factor Xa. $k_{obs}$ values measured as a function of unlabeled antithrombin-heparin binary complex were fit by the equation for competitive binding kinetics (solid lines) as detailed under "Experimental Procedures." Error bars indicate S.D. from the mean of at least five measured values.

FIGURE 10. Reaction scheme for exosite promotion of heparin-antithrombin-protease reactions. The binding of heparin (thick black curved line) to antithrombin (gray oval with white arrows representing the strands of β-sheet A and a thin curved line representing the reactive center loop) activates the serpin by generating an exosite (hook-shaped striped line). This exosite promotes the formation of a Michaelis complex between the antithrombin reactive loop and the active site of factors Xa or IXa (solid bean-shaped object) by engaging a complementary exosite on the protease (striped hook-shaped line) that is absent in thrombin. An additional heparin bridging exosite engages a basic exosite on factor Xa/IXa as well as on thrombin in the Michaelis complex. The stabilization of the Michaelis complex by exosite interactions favors the subsequent acylation step ($k_2 > k_1$) in which the protease active site serine becomes covalently attached to the reactive loop P1 residue and the P1-P1′ bond is cleaved. As a result, the overall reaction becomes limited by the rate of diffusional encounter of serpin and protease ($k_5$). The exosite interactions in the acyl-intermediate complex must subsequently be disrupted ($k_8$) for the serpin to undergo the conformational change ($k_9$) that traps the protease in the acyl-intermediate complex and causes heparin to dissociate. The trapping results from the cleaved reactive loop inserting into sheet A and dragging the attached protease to the bottom of the sheet where it is inactivated by deformation. The breaking of the exosite interactions following the acylation step limits the rate of the serpin conformational change step ($k_8$). As a result, a fraction of the acyl-intermediate escapes trapping by undergoing deacylation ($k_9$) through a competing substrate pathway that produces active protease and cleaved serpin.
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Exosites cause the rate of antithrombin-protease reactions to become limited by the rate of diffusional encounter of serpin and protease. Because of this change in rate-determining step, heparin enhancements in $k_{on}$ will not accurately reflect heparin enhancements of the Michaelis complex affinity. Thus, as long as there is sufficient binding energy provided by exosite residues in making progressively lesser although significant contributions to the interaction in keeping with the observation that these residues make contacts with factor Xa in the X-ray structure of the Michaelis complex (27).

Conclusions—Together the present studies support our previously proposed reaction model to explain how exosites promote the reactions of serpins with their target proteases (38). In this model exosite interactions act to promote the formation of the initial Michaelis docking complex between serpin and protease by enhancing the rate of complex formation ($k_1$) and decreasing the rate of complex dissociation ($k_{-1}$) (Fig. 10). The reduced off-rate constant favors the forward acylation reaction of the Michaelis complex ($k_2$), causing the overall reaction to become limited by the on-rate constant ($k_1$). In the case of antithrombin, the exosites that stabilize the Michaelis complex are generated on antithrombin and provided by a bridging heparin when heparin binds and conformationally activates the serpin. The exosites on heparin-activated antithrombin engage complementary exosites on thrombin and factor Xa in the Michaelis complex (10, 11, 21–23, 28, 29, 48). The specificity of these exosite interactions is supported by the observation that heparin only marginally promotes the Michaelis complex interaction or accelerates antithrombin inhibition of the related protease, trypsin (47). The increased stabilization of the Michaelis complex by exosites was shown to have a negative effect on the subsequent serpin conformational change that leads to trapping of the acyl-intermediate complex in the case of the plasminogen activator inhibitor-1 reaction with tissue-type plasminogen activator (38). This is because of the increased number of interactions that must be disrupted following acylation and cleavage of the reactive loop in the complex to allow the trapping serpin conformational change to occur. Evidence that the rate-limiting step beyond the Michaelis complex was the disruption of exosite interactions between the protease and the serpin in the acyl-intermediate complex to allow the serpin conformational change was provided in this case. In the conformational change, the cleaved reactive loop and acyl-linked protease inserts into $\beta$-sheet A, dragging the protease to the bottom of the sheet where it is catalytically disabled by deforming the active site (3, 4, 43). That a similar rate-limiting disruption of exosite interactions in the acyl-intermediate complex occurs in heparin-promoted antithrombin-protease reactions is suggested by the observation that the engagement of antithrombin or heparin bridging exosite interactions or the strengthening of heparin bridging exosite interactions by lowering the ionic strength in the acyl-intermediate complex results in increased deacylation rather than conformational trapping of the acyl-intermediate. Such increased deacylation is evident from an increased flux through the substrate pathway (Table 1) and corresponding decreases in $k_{lim}$ (7, 17, 48–50). The disruption of heparin bridging exosite interactions that occurs following the serpin conformational change promotes the dissociation of heparin and accounts for the catalytic action of the polysaccharide effector (49, 50). The observation that many serpins utilize exosites to recognize their target proteases (2) suggests that this reaction scheme may provide a general model for physiologic serpin-protease reactions.

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REFERENCES

1. Björk, I., and Olson, S. T. (1997) in Chemistry and Biology of Serpins (Church, F. C., Cunningham, D. D., Ginsburg, D., Hoffman, M., Stone, S. R., and Tollesen, D. M., eds) pp. 17–33, Plenum Press, New York

2. Gettins, P. G. W. (2002) Chem. Rev. 102, 4751–4803

3. Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) Nature 407, 923–926

4. Dementiev, A., Dobó, J., and Gettins, P. G. W. (2006) J. Biol. Chem. 281, 3452–3457

5. Olson, S. T., Swanson, R., Raub-Segall, E., Bedsted, T., Sadri, M., Petitzou, M., Herault, J.-P., Herbert, J.-M., and Björk, I. (2004) Thromb. Haemostasis 92, 929–939

6. Chooay, J., Petitzou, M., Lormeau, J. C., Sinay, P., Casu, B., and Gatti, G. (1983) Biochem. Biophys. Res. Commun. 116, 492–499

7. Olson, S. T., Björk, I., Shaffer, R., Craig, P. A., Shore, I. D., and Chooay, J. (1992) J. Biol. Chem. 267, 12528–12538

8. Olson, S. T., Srinivasan, K. R., Björk, I., and Shore, J. D. (1981) J. Biol. Chem. 256, 11073–11079

9. Olson, S. T., and Björk, I. (1991) J. Biol. Chem. 266, 6353–6364

10. Dementiev, A., Petitzou, M., Herbert, J.-M., and Gettins, P. G. W. (2004) Nat. Struct. Mol. Biol. 11, 863–867

11. Li, W., Johnson, D. J. D., Esonm, C. T., and Huntington, J. A. (2004) Nat. Struct. Mol. Biol. 11, 857–862

12. Rezaie, A. R. (1998) J. Biol. Chem. 273, 16824–16827

13. Danielsen, A., Raub, E., Lindahl, U., and Björk, I. (1986) J. Biol. Chem. 261, 15467–15473

14. Jin, L., Abrahams, J. P., Skinner, R., Petitzou, M., Pike, R. N., and Carrell, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14683–14688

15. Skinner, R., Abrahams, J. P., Whistock, J. C., Lesk, A. M., Carrell, R. W., and Wardell, M. R. (1997) J. Mol. Biol. 266, 601–609

16. Pike, R. N., Potempa, J., Skinner, R., Fittin, H. L., McGraw, W. T., Travis, J., Owen, M., Jin, L., and Carrell, R. W. (1997) J. Biol. Chem. 272, 19652–19655

17. Chuang, Y.-J., Gettins, P. G. W., and Olson, S. T. (1999) J. Biol. Chem. 274, 28142–28149

18. Chuang, Y.-J., Swanson, R., Raja, S. M., and Olson, S. T. (2001) J. Biol. Chem. 276, 14961–14971

19. Chuang, Y.-J., Swanson, R., Raja, S. M., Bock, S. C., and Olson, S. T. (2001) Biochemistry 40, 6670–6679
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20. Bedsted, T., Swanson, R., Chuang, Y.-J., Bock, P. E., Björk, I., and Olson, S. T. (2003) Biochemistry 42, 8143–8152
21. Yang, L., Manithody, C., and Rezaie, A. R. (2002) J. Biol. Chem. 277, 50756–50760
22. Rezaie, A. R. (2000) J. Biol. Chem. 275, 3320–3327
23. Sheehan, J. P., and Sadler, J. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5518–5522
24. Izaguirre, G., Zhang, W., Swanson, R., Bedsted, T., and Olson, S. T. (2003) J. Biol. Chem. 278, 51433–51440
25. Izaguirre, G., and Olson, S. T. (2006) J. Biol. Chem. 281, 13424–13432
26. Backovic, M., and Gettins, P. G. W. (2002) J. Proteome Res. 1, 367–373
27. Johnson, D. J. D., Li, W., Adams, T. E., and Huntington, J. A. (2006) EMBO J. 25, 2029–2037
28. Manithody, C., Yang, L., and Rezaie, A. R. (2002) Biochemistry 41, 6780–6788
29. Rezaie, A. R., Yang, L., and Manithody, C. (2004) Biochemistry 43, 2898–2905
30. Olson, S. T., Björk, I., and Shore, J. D. (1993) Methods Enzymol. 222, 525–560
31. Turk, B., Brieditis, I., Bock, S. C., Olson, S. T., and Björk, I. (1997) Biochemistry 36, 6682–6691
32. Nordenman, B., Nyström, C., and Björk, I. (1977) Eur. J. Biochem. 78, 195–203
33. Miletich, J. P., Broze, G. J., Jr., and Majerus, P. W. (1981) Methods Enzymol. 80, 221–228
34. Owen, W. G., and Jackson, C. M. (1973) Thromb. Res. 3, 705–714
35. Rezaie, A. R. (2006) Biochemistry 45, 5324–5329
36. Rezaie, A. R. (1996) Biochemistry 35, 1918–1924
37. Petitou, M., Herault, J., Bernat, A., Driguez, P., Duchaussay, P., Lormeau, I., and Herbert, J. (1999) Nature 398, 417–422
38. Olson, S. T., Swanson, R., Day, D., Verhamme, I., Kvassman, J., and Shore, J. D. (2001) Biochemistry 40, 11742–11756
39. Lindahl, P., Raub-Segall, E., Olson, S. T., and Björk, I. (1991) Biochem. J. 276, 387–394
40. Swanson, R., Raghavendra, M. P., Zhang, W., Froelich, C., Gettins, P. G. W., and Olson, S. T. (2007) J. Biol. Chem. 282, 2305–2313
41. Miller, D. M., III, Olson, J. S., and Quiocho, F. A. (1980) J. Biol. Chem. 255, 2465–2471
42. Sun, X. J., and Chang, J.-Y. (1989) J. Biol. Chem. 264, 11288–11293
43. Stratikos, E., and Gettins, P. G. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4808–4813
44. Jordan, R. E., Kilpatrick, J., and Nelson, R. M. (1987) Science 237, 777–780
45. Shore, J. D., Day, D. E., Francis-Chmura, A. M., Verhamme, I., Kvassman, J., Lawrence, D. A., and Ginsburg, D. (1995) J. Biol. Chem. 270, 5395–5398
46. Stone, S. R., and Le Bonniec, B. F. (1997) J. Mol. Biol. 265, 344–362
47. Olson, S. T., Bock, P. E., Kvassman, I., Shore, J. D., Lawrence, D. A., Ginsburg, D., and Björk, I. (1995) J. Biol. Chem. 270, 30007–30017
48. Rezaie, A. R., and Olson, S. T. (2000) Biochemistry 39, 12083–12090
49. Olson, S. T. (1985) J. Biol. Chem. 260, 10153–10160
50. Craig, P. A., Olson, S. T., and Shore, J. D. (1989) J. Biol. Chem. 264, 5452–5461