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COOPERATIVE INTERACTIONS OF THREE HOTSPOT HEPARIN BINDING RESIDUES ARE CRITICAL FOR ALLOSTERIC ACTIVATION OF ANTITHROMBIN BY HEPARIN

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Abstract

Heparin allosterically activates the anticoagulant serpin, antithrombin, by binding through a sequence-specific pentasaccharide and inducing activating conformational changes in the protein. Three basic residues of antithrombin, Lys114, Lys125 and Arg129, have been shown to be hotspots for binding the pentasaccharide, but the molecular basis for such hotspot binding has been unclear. To determine whether this results from cooperative interactions, we analyzed the effects of single, double and triple mutations of the hotspot residues on pentasaccharide binding and activation of antithrombin. Double mutant cycles revealed that the contribution of each residue to pentasaccharide binding energy was progressively reduced when one or both of the other residues were mutated, indicating strong coupling between each pair of residues that was dependent on the third residue and reflective of the three residues acting as a cooperative unit. Rapid kinetic studies showed that the hotspot residue mutations progressively abrogated both the ability of the pentasaccharide to bind productively to native antithrombin and to conformationally activate the serpin by engaging the hotspot residues in an induced-fit interaction. Examination of the antithrombin-pentasaccharide complex structure revealed that the hotspot residues form two adjoining binding pockets for critical sulfates of the pentasaccharide which structurally link these residues. Together, these findings demonstrate that cooperative interactions of Lys114, Lys125 and Arg129 are critical for the productive induced-fit binding of the heparin pentasaccharide to antithrombin that allosterically activates the anticoagulant function of the serpin.

INTRODUCTION

Antithrombin is a key anticoagulant protein of the serpin superfamily that regulates the activity of several serine proteases of the blood coagulation cascade in conjunction with its glycosaminoglycan cofactors, heparin and heparan sulfate, in vertebrates (1). The importance of antithrombin as an anticoagulant regulator of hemostasis is underscored by the increased risk of thrombotic disease in humans with acquired or inherited deficiencies of antithrombin (2) and by the embryonic lethality or severe coagulopathy resulting from complete deficiency in mice (3) or zebrafish (4). Antithrombin and its glycosaminoglycan

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cofactors function as potent anticoagulants by inhibiting coagulation proteases that are activated by vascular injury or other procoagulant stimuli. This inhibition is relatively slow in the absence of the glycosaminoglycans but is accelerated several thousand-fold when antithrombin binds heparin and heparan sulfate glycosaminoglycans that line the vascular wall or become exposed at sites of injury (5,6). The importance of heparin and heparan sulfate as anticoagulant activators of antithrombin is suggested by the association of inherited mutations of antithrombin that cause heparin binding defects with an increased susceptibility of affected individuals to thrombosis (2).

Heparin activates antithrombin inhibitory function by acting both as an allosteric activator of the serpin and as a bridging cofactor (7,8). In the allosteric mechanism, a sequence-specific pentasaccharide in a fraction of heparin or heparan sulfate chains acts as a receptor that binds antithrombin with high-affinity and conformationally activates the inhibitor to rapidly and specifically inhibit two target proteases, factor Xa and factor IXa (8–10). In the bridging mechanism, heparin binding of both antithrombin and the protease serves to localize the proteins on the linear glycosaminoglycan chain and promote their interaction. This latter mechanism greatly enhances antithrombin reactivity with thrombin and augments antithrombin reactivity with factors Xa and IXa (11,12).

Studies of natural and engineered mutants of antithrombin with defects in heparin binding (13) together with X-ray structures of free and heparin-complexed antithrombin (14,15) have demonstrated the site of heparin binding and the conformational alterations that accompany this binding and act to enhance antithrombin reactivity with factors Xa and IXa. The heparin binding site is centered on helix **D** and includes the N-terminal tail and helix A, regions which are closely associated in the tertiary structure of the protein. Heparin pentasaccharide binding to this site triggers conformational changes that position basic and polar residues for an induced-fit interaction with the pentasaccharide (14). These localized conformational changes are transmitted through the hydrophobic core to result in i) expulsion of the reactive center loop (RCL)¹ from an intramolecular interaction with sheet A and ii) an altered juxtaposition of the RCL with an exosite in sheet C (16–18). The latter changes enhance the interactions of factor Xa and factor IXa with the RCL and sheet C exosite of antithrombin through the active-sites and conserved basic residues in the autolysis loops of the proteases that are absent in thrombin (19,20).

Three highly conserved heparin binding residues located on helix D and the loop preceding this helix, Lys114, Lys125 and Arg129, have been shown to be hotspots in antithrombin for binding the heparin pentasaccharide receptor sequence and inducing conformational activation (21–24) (Figure 1). Individual mutations of these residues result in massive losses in heparin pentasaccharide binding energy (25–50%) that greatly weaken the induced-fit interaction of the pentasaccharide with the activated state which drives allosteric activation. Other basic and nonbasic residues in the N-terminal tail and on helix A also contribute to binding the pentasaccharide, but mutation of these residues results in much smaller losses of pentasaccharide binding energy and modest effects on the induced-fit interaction with the activated state (26,27).

The present study was designed to determine whether the critical roles of the hotspot residues in heparin binding and allosteric activation of antithrombin derive from their cooperative action. Cooperative interactions among the three residues were assessed from the effects of single, double and triple mutations on pentasaccharide binding and activation (28,29). Our findings reveal nonadditive losses in pentasaccharide binding energy resulting from combined mutations of the three hotspot residues that are indicative of strong cooperative interactions. Most notably, rapid kinetic studies show that the cooperative interactions of the hotspot residues are critical to electrostatically guide the pentasaccharide to a productive binding site on antithrombin and to induce a complementary high-affinity interaction with the bound pentasaccharide that activates antithrombin. A high resolution structure of the antithrombin-pentasaccharide complex (18) shows that the hotspot residues are key elements of two structurally linked binding pockets that interact with critical sulfates of the pentasaccharide, suggesting a structural basis for the cooperative action of the hotspot residues in mediating allosteric activation of antithrombin by the heparin pentasaccharide.

MATERIALS AND METHODS

Proteins.

Recombinant wild-type and variant antithrombins with single, double and triple mutations of Lys114, Lys125 and Arg129 to Met were engineered on a wild-type β -antithrombin background in which the Asn135 glycosylation site was mutated to Gln to block glycosylation (30). A 6XHis-tag was inserted at the C-terminal end of all recombinant proteins by PCR mutagenesis of the wild-type antithrombin cDNA in the plasmid, pcDNA 3.1(+). Mutations were introduced into the His-tagged antithrombin cDNA after cloning into the pFASTBac baculovirus plasmid (Invitrogen) by PCR using specifically designed nucleotides as described previously (31). All mutations were confirmed by DNA sequencing. Recombinant proteins were expressed in Sf9 insect cells by transfecting with bacmid vectors containing the mutant antithrombin cDNA as described (32). Variants were purified from culture media by chromatography on a 5 mL HisPur Ni-NTA affinity column (ThermoFisher Scientific). After concentration and centrifugation of the media, it was buffer exchanged into 20 mM Hepes, 50 mM NaCl, 20 mM imidazole buffer, pH 7.5 by ultrafiltration and loaded on the column equilibrated in this buffer. The column was washed with 10 column volumes buffer containing 1 M NaCl and then restored to equilibrating buffer. Antithrombins were eluted with a gradient up to 500 mM imidazole in the Hepes equilibrating buffer. The column eluate was monitored for protein with a fluorescence detector set at λ_{ex} 280 nm, λ_{em} 340 nm. Protein peaks were pooled, concentrated and buffer exchanged into 0.01–0.1 M Hepes, 0.1 M NaCl, pH 7.4. Further purification on a MonoQ column was done as previously described with some variants (22). No attempt was made to remove the His-tag since all functional properties of the tagged wild-type antithrombin investigated were found to be indistinguishable from those of the untagged protein (33). Concentrations of recombinant antithrombins were determined from the 280 nm absorbance using an extinction coefficient of 37,700 M⁻¹cm⁻¹ (34).

Human thrombin was purchased from US Biochemical Corp. and factor Xa and factor IXa were purchased from Enzyme Research Labs. Active protease concentrations were

determined from standard substrate assays that were calibrated with active-site titrated enzymes (9,35).

Heparin.

The synthetic natural pentasaccharide (fondaparinux) representing the antithrombin binding sequence in heparin and a “super” pentasaccharide containing an additional 3-O-sulfate on the reducing end saccharide (idraparinux) (10,36) were generously provided by Sanofi-Aventis (Toulouse, France) or purchased from the University of Illinois Hospital pharmacy. Concentrations of pentasaccharides were determined by stoichiometric binding titrations of plasma antithrombin with the saccharides monitored from the tryptophan fluorescence enhancement that accompanies binding as described (37).

SDS-PAGE.

The purity of recombinant antithrombins and their ability to form complexes with thrombin was analyzed by SDS-PAGE on 10% acrylamide gels with the Laemmli buffer system (38). ~3 μ g antithrombin without or with a slight molar excess of thrombin was incubated for 10–20 min in 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4, I 0.15 at 25°C, residual thrombin inactivated with ~ 100-fold molar excess of FPR-chloromethylketone and then the proteins were denatured by boiling in SDS buffer under reducing conditions prior to electrophoresis.

Stoichiometries of antithrombin-protease reactions.

Antithrombin inhibitory function was analyzed by stoichiometric titrations of 50–100 nM thrombin with up to a ~2-fold molar excess of antithrombin in I 0.15 sodium phosphate buffer, pH 7.4 (see above) at 25°C. Following incubation of 100 μ l antithrombin-thrombin mixtures for a time sufficient to achieve complete inhibition, 900 μ l 100 μ M S2238 chromogenic substrate in the same buffer was added and the residual thrombin activity determined by following the initial linear rate of substrate hydrolysis from the absorbance increase at 405 nm. Linear regression analysis of the decrease in thrombin activity as a function of the antithrombin/thrombin molar ratio yielded the stoichiometry from the x-axis intercept. Functional antithrombin concentrations of wild-type and variant antithrombins were typically checked prior to analyses of heparin pentasaccharide binding or kinetic analyses of antithrombin inhibition of factor Xa and factor IXa as described below.

Binding of heparin pentasaccharides to antithrombin.

The affinity of pentasaccharides for wild-type and variant antithrombins was analyzed by titrations of ~100 nM antithrombin with the pentasaccharides monitored by changes in tryptophan fluorescence (λ_{ex} 280 nm, λ_{em} 340 nm) which report pentasaccharide binding with an SLM 8000 spectrofluorimeter as in previous studies (8,37). Titrations were conducted in 10–20 mM sodium phosphate buffer, containing 0.1 mM EDTA, 0.1% PEG 8000 and 0–1 M NaCl adjusted to pH 6.0 to achieve a range of ionic strengths at 25°C. Antithrombin variants were extensively diluted from concentrated stock solutions (>10 μ M) to ensure that the pH and ionic strength of buffers was minimally perturbed. Observed

fluorescence changes as a function of added pentasaccharide were corrected for dilution (<5%) and computer fit by nonlinear regression by the quadratic binding equation:

$$F_{\text{obs}} = F_0 + \Delta F_{\text{max}} \times ([H]_0 + n[AT]_0 + K_{D,\text{obs}} - (([H]_0 + n[AT]_0 + K_{D,\text{obs}})^2 - 4[H]_0 n[AT]_0)^{1/2}) / (2n[AT]_0)$$

Equation 1

where F_{obs} is the observed fluorescence after each addition of heparin, F_0 is the initial fluorescence and ΔF_{max} is the maximal fluorescence change, $[H]_0$ and $[AT]_0$ are the total heparin pentasaccharide and antithrombin concentrations, respectively, n is the binding stoichiometry and $K_{D,\text{obs}}$ is the observed dissociation constant for the antithrombin-pentasaccharide interaction. F_0 , ΔF_{max} , n and $K_{D,\text{obs}}$ were the fitted parameters. Titrations in which antithrombin concentrations were much greater than $K_{D,\text{obs}}$ provided good fits of the binding stoichiometry which were indistinguishable from 1 as long as functional inhibitor concentrations based on stoichiometric titrations of thrombin activity were employed. To best determine $K_{D,\text{obs}}$, titrations were done at antithrombin concentrations close to or below $K_{D,\text{obs}}$, with n fixed at 1 in fits of these titrations. Heparin was added at concentrations of at least $5 \times K_{D,\text{obs}}$ in most cases to achieve near saturation of antithrombin.

Quantitation of antithrombin-pentasaccharide interactions by kinetic titrations.

The binding affinity of the heparin pentasaccharide for some antithrombin variants was measured by kinetic titrations of the variant with pentasaccharide wherein binding was monitored from the enhancement in the rate of antithrombin inhibition of factor Xa produced by pentasaccharide binding. Titrations were conducted at 25°C in 10–20 mM sodium phosphate, 0.1 mM EDTA, 0.1% PEG 8000, pH 6.0 buffer with the NaCl concentration varied from 0–0.015 M to achieve ionic strengths of 0.012–0.040. The kinetics of antithrombin inhibition of factor Xa as a function of added pentasaccharide up to at least $2 \times K_d$ and typically $>5 \times K_D$ were measured under pseudo-first order conditions with >10 -fold molar excess of inhibitor over protease for fixed reaction times that resulted in 20–80% factor Xa inhibition. Reactions (50 μ l) were quenched by adding 1 mL 100 μ M Spectrozyme FXa containing 100 μ g/mL Polybrene in 0.15 sodium phosphate buffer, pH 7.4 and the initial velocity of substrate hydrolysis measured at 405 nm. Apparent second order rate constants observed at each pentasaccharide concentration ($k_{\text{app,obs}}$) were calculated from the extent of factor Xa inhibition from the expression:

$$k_{\text{app,obs}} = -\ln(v_t/v_o)/([AT]_0 \times t) \quad \text{Equation 2}$$

where v_t and v_o are the initial velocities of factor Xa hydrolysis of substrate after reaction with inhibitor for time, t , and for a control reaction without inhibitor, respectively. $K_{D,\text{obs}}$ for the antithrombin-pentasaccharide interaction was determined by nonlinear regression fitting of the dependence of $k_{\text{app,obs}}$ on pentasaccharide concentration by the quadratic equation 1

above with F_{obs} , F_o and F_{max} replaced by $k_{app,obs}$, $k_{app,o}$ and $k_{app,max}$, where $k_{app,o}$ is the basal value of $k_{app,obs}$ in the absence of pentasaccharide and $k_{app,max}$ is the maximal change in $k_{app,obs}$ when antithrombin is fully complexed with pentasaccharide. $k_{app,o}$ was fit or fixed at independently determined values, n was fixed at 1 and $K_{D,obs}$ and $k_{app,max}$ were fitted parameters.

Kinetics of antithrombin inhibition of proteases in the absence and presence of pentasaccharide.

Second order association rate constants for antithrombin inhibition of proteases in the absence and presence of saturating pentasaccharide were measured under pseudo-first order conditions as in the kinetic titrations. Reactions of antithrombin with thrombin were done only in the absence of pentasaccharide in 20 mM sodium phosphate, 0.1 mM EDTA, 0.1% PEG 8000, pH 6.0 at 25°C. Reaction mixtures (100 μ l) containing fixed concentrations of antithrombin and thrombin were incubated for varying times and then quenched with 900 μ l 100 μ M S-2238 substrate in 10.15 sodium phosphate buffer, pH 7.4 and residual thrombin activity measured from the initial rate of substrate hydrolysis at 405 nm. Reactions of antithrombin with factor Xa or with factor IXa were conducted in 20 mM MES, 5 mM $CaCl_2$, 0.1% PEG 8000, pH 6.0 at 25°C in the absence and presence of the natural pentasaccharide. For reactions in the absence of pentasaccharide, fixed antithrombin and factor Xa or factor IXa concentrations were incubated for varying times or fixed protease and varying antithrombin concentrations were incubated for a fixed reaction time in 50–100 μ l and then quenched with 1 ml chromogenic substrate to measure residual protease activity from the initial rate of substrate hydrolysis at 405 nm. The factor Xa substrate was 100 μ M Spectrozyme FXa and the factor IXa substrate was 300 μ M Pefachrome FIXa, both in 0.1 M Hepes, 0.1 M NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4. Factor IXa substrate solutions were supplemented with 33% ethylene glycol and 10 mM $CaCl_2$ to enhance factor IXa activity (39). The loss in protease activity as a function of time or as a function of antithrombin concentration was fit by the exponential function:

$$v_{obs} = v_o \times \exp(-k_{-H} \times [AT]_o \times t) \quad \text{Equation 3}$$

where v_{obs} and v_o are the observed velocity of substrate hydrolysis by protease after inhibitor reaction and control velocity without inhibitor, respectively, and k_{-H} is the second order association rate constant for the free antithrombin reaction. Dividing the fitted exponential constant by the antithrombin concentration when time was varied or by the fixed time when the antithrombin concentration was varied then yielded k_{-H} .

For reactions in the presence of pentasaccharide, fixed concentrations of antithrombin, protease and saturating pentasaccharide were reacted for varying times or varying antithrombin concentrations were reacted with fixed concentrations of protease and saturating pentasaccharide for a fixed reaction time in 50–100 μ l and then quenched with 1 ml chromogenic substrate to measure residual protease activity at 405 nm. The loss of protease activity as a function of time or as a function of antithrombin concentration was fit by the exponential equation above with k_{-H} replaced by k_H , the second order association rate

constant for the antithrombin-heparin complex reaction. Dividing the fitted exponential constant by the antithrombin concentration when time was varied or by the fixed time when the antithrombin concentration was varied yielded k_H . Alternatively, fixed concentrations of antithrombin and protease were reacted with varying subsaturating pentasaccharide concentrations ($[H]_0 \ll [AT]_0$) for a fixed reaction time. Fitting the loss of protease activity as a function of pentasaccharide concentration by an exponential function in this case gives a fitted exponential constant that must be divided by the product of the fixed reaction time and the factor, $[AT]_0/(K_{D,obs} + [AT]_0)$, to correct for the fraction of antithrombin that is complexed with pentasaccharide so as to obtain k_H (19).

Rapid kinetics of heparin pentasaccharide binding to antithrombin.

The kinetics of pentasaccharide binding to wild-type and variant antithrombins were measured under pseudo-first order conditions, i.e., with at least a 5-fold molar excess of pentasaccharide over antithrombin, by monitoring antithrombin tryptophan fluorescence changes following the mixing of equal volumes of inhibitor and heparin as in past studies (8,40). The kinetics were studied in 20 mM sodium phosphate, 0.015 M NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 6.0, 0.04 at 25°C. Fluorescence changes reporting the binding interaction were monitored with an Applied Photophysics SX-17MV stopped-flow fluorimeter with λ_{ex} 280 nm and an emission cut-on filter with 50% transmittance at 320 nm. Progress curves obtained as a function of heparin pentasaccharide concentration were computer fit by a single exponential function to obtain the pseudo-first order binding rate constant, k_{obs} .

Antithrombin and heparin effects on TNS fluorescence.

Fluorescence spectra of the fluorophore probe, TNS, (λ_{ex} 330 nm, λ_{em} 350–550 nm) were measured at 10 μ M with the SLM 8000 spectrofluorimeter in the absence and presence of 500 nM antithrombin variants and after titrating the variants with heparin pentasaccharide to saturation in sodium phosphate buffer, I 0.025, pH 6.0 at 25°C. Spectra of TNS bound to antithrombin in the absence and presence of saturating pentasaccharide were obtained by subtracting the free TNS fluorescence spectrum based on the TNS concentration being well below K_D for binding to antithrombin (41). Spectra were corrected for buffer background and dilution and normalized to the maximal fluorescence of free TNS.

Thermal stabilities of antithrombin variants.

Thermal stabilities were measured by monitoring tryptophan fluorescence changes of the variants (λ_{ex} 280 nm, λ_{em} 340 nm) that accompany thermal denaturation as a function of increasing temperature in the range 40–75°C with an SLM 8000 spectrofluorimeter equipped with a Peltier temperature controller as in past studies (32,42). 100 nM antithrombin solutions in I 0.15 sodium phosphate buffer, pH 7.4 were equilibrated at 40°C and the initial fluorescence recorded for several 10 s intervals. The temperature was then increased in 5 degree intervals at a rate of 0.5°C/min and the fluorescence recorded at each interval after temperature equilibration. Solutions were continuously stirred during the titrations. The sigmoid-shaped denaturation curves were computer fit by the van't Hoff equation to obtain the melting temperature reflecting the midpoint of the transition between native and denatured states (32).

RESULTS

Engineering and properties of antithrombin variants.

The cooperative interactions of three hotspot heparin binding residues of antithrombin, Lys114, Lys125 and Arg129, in binding the heparin pentasaccharide and mediating allosteric activation of the serpin were investigated by engineering antithrombin variants in which these residues were mutated alone, pairwise and all three together to the isosteric Met. A C-terminal His-tag was added to all variants to allow their purification by nickel affinity chromatography because the traditional purification method of heparin affinity chromatography was not considered feasible given the anticipated heparin binding defects of the variants. All variants could be purified to homogeneity and were capable of forming SDS-stable equimolar complexes with thrombin when analyzed by SDS-PAGE (Figure 2).

Stoichiometries of thrombin inhibition by the antithrombin variants measured in the absence of heparin by endpoint titrations revealed that all but two inhibited thrombin with stoichiometries close to 1:1 (1.0–1.3 moles antithrombin/mole protease). The variants, K125M and K125M/R129M, exhibited higher thrombin inhibition stoichiometries approaching 2 moles antithrombin/mole thrombin and also bound the heparin pentasaccharide activator with corresponding binding stoichiometries approaching 0.5 mole heparin/mole antithrombin. These variants thus appeared to contain nonfunctional, most likely latent antithrombin, as noted in previous studies (21–23). Second order rate constants measured for thrombin inhibition by the antithrombin variants after correction for nonfunctional protein yielded values essentially indistinguishable from wild-type with or without a His-tag (Table 1). Neither the heparin binding site mutations nor the His tag thus affected antithrombin inhibitory function in the absence of heparin. Analysis of thermal titrations of the antithrombin variants showed that the Lys125 single and Lys125/Arg129 double mutants exhibited melting temperatures ~2–4°C less than wild-type whereas all other variants exhibited melting temperatures equal to or up to 3°C greater than wild-type (Table 1). The mutations thus caused differential effects on serpin stability that was likely responsible for the variable levels of nonfunctional protein.

Heparin binding to the antithrombin variants

The effect of the heparin binding site mutations on antithrombin affinity for the sequence-specific heparin pentasaccharide activator of the serpin was analyzed by equilibrium binding titrations monitored by the ~40% enhancement of tryptophan fluorescence that reports pentasaccharide binding and conformational activation of antithrombin (44). Because of the wide range of heparin affinities anticipated for the variants and need to compare affinities under a single set of conditions, heparin binding titrations were performed at pH 6 to enhance the heparin affinity of weak binding variants and over a range of ionic strengths to allow heparin affinities of tighter binding variants to be accurately measured and assessed at lower ionic strength by extrapolation (43). Heparin affinities were measured both for the natural heparin pentasaccharide as well as for a higher affinity “super” pentasaccharide with an additional sulfate on the reducing end residue (36). Both pentasaccharides produce equivalent activating effects on antithrombin anticoagulant function (11). All except two variants, the double mutant, K114M/K125M, and the triple K114M/K125M/R129M mutant

(designated MMR and MMM), produced significant fluorescence changes upon pentasaccharide binding that enabled heparin affinities to be accurately measured by fluorescence titrations (Table 1). The MMR and MMM variants did produce significant enhancements in reactivity with factor Xa upon pentasaccharide binding that enabled the heparin affinity of these variants to be measured by kinetic titrations (Figure 3). The binding affinities measured from such kinetic titrations were validated from the finding that affinities measured for other variants by both equilibrium and kinetic titrations yielded indistinguishable values (Table 3).

The ionic strength dependence of observed dissociation constants for heparin pentasaccharide binding to the antithrombin variants ($K_{D,obs}$) was analyzed based on the equation derived from polyelectrolyte theory (45),

$$\log K_{D,obs} = Z\Psi \log[Na^+] + \log K_D' \quad \text{Equation 4}$$

where Z is the number of ionic interactions mediating the binding, Ψ is the fraction of sodium ions displaced from heparin by the binding of antithrombin (equal to 0.8) (8) and K_D' represents the dissociation constant for the nonionic contribution to the binding, i.e., hydrogen bonding, hydrophobic and van der Waals interactions. All variants showed linear increases in $\log K_{D,obs}$ as a function of $\log [Na^+]$ for the binding of both natural and super pentasaccharides (Figure 4), in accordance with this equation and consistent with previous studies of antithrombin-heparin interactions (8). The slopes and intercepts of these plots provided Z and $\log K_D'$, respectively, for each interaction (Table 2).

Natural and super pentasaccharides bound wild-type antithrombin with ~6 and ~7 ionic interactions, respectively, consistent with previous studies showing that the additional sulfate on the super pentasaccharide contributes one more ionic interaction (46). The single mutation of Lys114 (MKR) caused the loss of 3 ionic interactions with the natural pentasaccharide and 3–4 ionic interactions with the super pentasaccharide whereas single mutations of Lys125 (KMR) and Arg129 (KKM) each caused the loss of ~2 ionic interactions in binding either the natural or super pentasaccharide. Combining the Lys114 mutation with Lys125 or Arg129 in MMR and MKM double mutants or with both residues in the MMM triple mutant all resulted in the loss of ~4 ionic interactions with the natural pentasaccharide, i.e., less than that expected from the additive effects of the single mutants. Similarly, combining the Lys125 and Arg129 mutations in the KMM double mutant resulted in no additional losses of ionic interactions with the natural pentasaccharide, i.e., ~2, compared to either of the single mutants, indicating nonadditive effects of combining these mutations. The more limited data for the super pentasaccharide interactions showed that the KMM double mutation produced similar nonadditive losses in ionic interactions relative to single mutations whereas the MKM double mutation caused losses that were closer to additive. Most notably, Lys114 single and double mutants showed a loss of ~1 more ionic interaction with the super pentasaccharide than with the natural pentasaccharide whereas Lys125 and Arg129 single and double mutants showed the same loss of ionic interactions with natural and super pentasaccharides. This suggested that the Lys114 mutation disrupts the interaction with the additional sulfate of the super pentasaccharide.

The heparin binding site mutations caused a loss not only of ionic interactions but also of nonionic interactions (Table 2). These presumably derive mostly from the loss of hydrogen bonding interactions of the amine and guanidine moieties of Lys and Arg residues since the isosteric substitution of Met was expected to retain the nonpolar interactions of these sidechains. For antithrombin interactions with the natural pentasaccharide, the single mutations resulted in a loss of 55% of the nonionic binding energy of wild-type in the case of the Lys114 mutation and smaller losses of 24% and 17% for Lys125 and Arg129 mutations, respectively. Double mutations of Lys114/Lys125, Lys114/Arg129 or Lys125/Arg129 produced nonadditive losses of nonionic binding energy of 74%, 64% and 66% relative to wild-type, respectively. The triple mutant resulted in a nonionic binding energy loss of 60%, somewhat less than the losses of the three double mutants. The more limited data for antithrombin mutant interactions with the super pentasaccharide revealed nonionic binding energy losses due to single Lys125 or Arg129 mutations or the double Lys125/Arg129 mutation that approximated those observed for the natural pentasaccharide interactions. By contrast, the nonionic binding energy losses resulting from mutations of Lys114 in single or double mutant antithrombins were much greater for the super pentasaccharide interactions than the natural pentasaccharide interactions, suggesting that the additional sulfate of the super pentasaccharide contributed significant nonionic as well as ionic binding energy to the interaction.

Cooperativity of Lys114, Lys125 and Arg129 in binding heparin

The extent to which the three hotspot heparin binding residues cooperate in binding the heparin pentasaccharide was assessed from the binding energy losses caused by the single, double and triple mutations of the three residues relative to wild-type at I 0.025, pH 6, 25°C based on the ionic strength dependence of the binding (Table 3). Relative to the wild-type binding energy of 19.0 kcal/mole for the natural pentasaccharide interaction, the single mutation of Lys114 caused the largest loss of binding energy (9.7 kcal/mole) whereas single mutations of Lys125 or Arg129 produced smaller but still major losses of binding energy of 5.6 and 4.6 kcal/mole, respectively, consistent with previous studies of similar single mutants (21–23). The double mutations, Lys114/Lys125, Lys114/Arg129 and Lys125/Arg129 increased the binding energy losses of the single mutants to 12.3, 12.0 and 8.3 kcal/mole, respectively, indicating large nonadditive effects of these mutations. Remarkably, mutation of all three residues resulted in a loss in binding energy of 11.6 kcal/mole that was somewhat less than the losses caused by the double mutations of Lys114/Lys125 or Lys114/Arg129, but significantly greater than the loss caused by the Lys125/Arg129 double mutation.

The binding energy losses of the mutants were similarly analyzed for the super pentasaccharide interaction assuming that the double Lys114/Lys125 mutant and the triple mutant bound the super pentasaccharide with an affinity equal to that of the natural pentasaccharide, a reasonable assumption since the affinities of other Lys114 mutants for the two pentasaccharides were normalized (Table 3). Because Lys114 mediates the interaction with the additional sulfate of the super pentasaccharide, the wild-type binding energy was considerably greater for the super pentasaccharide than for the natural pentasaccharide interaction and single mutations caused a greater binding energy loss for the Lys114

mutation than for mutations of Lys125 or Arg129. As with the natural pentasaccharide interaction, double mutations increased the binding energy losses relative to single mutations in a less than additive manner and the triple mutations caused a further nonadditive loss in binding energy relative to the Lys125/Arg129 double mutations but a somewhat reduced binding energy loss relative to Lys114/Lys125 and Lys114/Arg129 double mutations. Double mutations of Lys114 with Lys125 or Arg129 are thus sufficient to maximize the loss in pentasaccharide binding energy for either natural or super pentasaccharides.

The nonadditivity of the binding energy losses observed with the double and triple mutants relative to the single mutants indicated strong cooperative interactions exist among the three heparin binding residues. The cooperative interactions are quantitatively defined by the coupling or interaction free energies between each pair of residues. These are calculated from double mutant cycles as the difference in binding energy losses when one residue of a pair is mutated in the context of a wild-type or mutant second residue (Figure 5) (28,29). The coupling between each pair of residues may additionally be affected by whether the third residue is wild-type or mutated. Mutation of the three hotspot residues in all possible sequences thus defines six double mutant cycles represented by the six faces of a cube (Figure 5) from which coupling energies can be derived for each pair of residues in the context of wild-type or mutant third residues.

Table 4 shows that for each of the three hotspot residues, the binding energy losses are the greatest when the two other residues are wild-type but are significantly reduced in the context of single mutations of the other two residues and further reduced in the context of mutations of both other residues. Notably, mutations of Lys125 or of Arg129 in the context of mutations of the other two residues show that these residues make a small negative contribution to pentasaccharide binding energy when cooperative interactions with other hotspot residues are eliminated. By contrast, mutation of Lys114 in the context of mutations of the other two residues show that Lys114 makes a modest residual contribution to pentasaccharide binding energy of one-third to one-half that in wild-type when cooperative interactions are abolished.

The derived coupling energies for the three pairs of hotspot residues in the context of wild-type and mutant third residues are shown in Table 5. Notably, the coupling energies for both pentasaccharide interactions represent a major fraction of Lys125 and Arg129 binding energies and a more modest fraction of Lys114 binding energy even though coupling energies for the super pentasaccharide interaction exceed those of the natural pentasaccharide interaction. Lys125 and Arg129 interactions with the pentasaccharide are thus more strongly coupled to those of Lys114 (50–66%) and to those of each other (34–51%) relative to the reciprocal coupling of Lys114 interactions (19–31%). Notably, the coupling energies are increased significantly by 1.1 kcal/mole for interactions with the natural pentasaccharide but more modestly by 0.2 kcal/mole for interactions with the super pentasaccharide in the context of a mutant third residue. All three pairs of residues thus exhibit a strong coupling that is dependent on the third residue, demonstrating that the three residues function as a cooperative unit (29).

Rapid kinetics of variant antithrombin interactions with heparin

The heparin pentasaccharide binds and conformationally activates antithrombin by an induced-fit binding mechanism in which an initial low-affinity binding of the pentasaccharide to native antithrombin induces an activated conformational state that binds the pentasaccharide with much higher affinity and thereby stabilizes the serpin in this state (8,43). This mechanism can be depicted by the scheme,

where AT is native antithrombin, H is heparin, AT.H is the native antithrombin-heparin complex, AT*.H is the activated antithrombin-heparin complex, K_1 is the apparent dissociation constant for the initial binding step and k_{+2} and k_{-2} are the forward and reverse rate constants for the conformational activation step, respectively. To determine how the three hotspot heparin binding residues of antithrombin cooperate to preferentially bind the pentasaccharide in the activated state, we performed rapid kinetic studies of heparin pentasaccharide binding to wild-type and mutant antithrombins under pseudo-first order conditions ($[\text{H}]_0 \gg [\text{AT}]_0$) at I 0.04, pH 6, 25°C monitored by the tryptophan fluorescence enhancement that accompanies binding. The two mutants that did not yield significant fluorescence changes upon pentasaccharide binding were not analyzed. The observed pseudo-first order rate constant for heparin binding to antithrombin (k_{obs}) by the induced-fit mechanism is expected to increase saturably with increasing heparin concentration according to the hyperbolic equation (40):

$$k_{\text{obs}} = k_{-2} + k_{+2} \times [\text{H}]_0 / (K_1 + [\text{H}]_0) \quad \text{Equation 5}$$

Single mutations of Lys125 or Arg129 or the double Lys125/Arg129 mutant all showed saturable increases in k_{obs} as the heparin concentration was increased similar to wild-type that were well fit by the hyperbolic equation (Figure 6). The fits indicated limiting rate constants at heparin saturation, reflecting the sum of forward and reverse conformational change rate constants ($k_{+2} + k_{-2}$), that were indistinguishable from wild-type for the Arg129 mutant (1020 s^{-1}) and at most 2-fold reduced from wild-type in the case of Lys125 single or Lys125/Arg129 double mutants ($550\text{--}690 \text{ s}^{-1}$) (Table 6). The reverse conformational change rate constants obtained from the extrapolated intercept of k_{obs} values at low heparin concentration were not well-defined, but consistent with a small value indistinguishable from zero for wild-type and single mutants and a more substantial value of $\sim 70 \text{ s}^{-1}$ for the double mutant. This indicated a robust conformational activation of these mutants that overwhelmingly favored the activated over the native conformation ($k_{+2}/k_{-2} \gg 1$). Saturation required increasingly higher heparin pentasaccharide concentrations for the single and double mutants than wild-type, reflecting a progressively weaker initial interaction of the pentasaccharide with native antithrombin that was most marked for the double mutant (K_1 values ranging from $\sim 2\text{--}30$ -fold greater than wild-type). Nevertheless, overall $K_{\text{D,obs}}$ values were markedly lower than K_1 for these mutants, consistent with the initial binding

interaction undergoing a major tightening as a result of the favorable conformational activation equilibrium (Table 6). This was reflected in values of K_2 calculated from K_1 and $K_{D,obs}$. Such values provide the most reliable measures of K_2 since k_{-2} inferred from extrapolated values of k_{obs} at low heparin concentration can reflect contributions of an alternative conformational selection pathway of conformational activation as noted below (21,23)

Contrasting these results, the single mutation of Lys114 or double mutation of Lys114 and Arg129 dramatically reduced the limiting rate constant reflecting conformational activation. The single Lys114 mutation showed a saturable increase in k_{obs} with a greatly reduced limiting rate constant of 40 s^{-1} reflecting the sum, $k_{+2} + k_{-2}$, and a poorly defined, modestly weakened affinity for the initial binding interaction (Figure 6 & Table 6). A significantly elevated reverse conformational activation rate constant was evident from the intercept value of k_{obs} ($\sim 20 \text{ s}^{-1}$), which implied a forward conformational activation rate constant of comparable value ($\sim 20 \text{ s}^{-1}$). The observation that $K_{D,obs}$ was ~ 10 -fold less than K_1 was consistent with the conformational activation equilibrium favoring the activated state and was in keeping with a minor reduction in the tryptophan fluorescence enhancement, consistent with previous studies of this mutant (22,33). Notably, the double Lys114/Arg129 mutant bound the heparin pentasaccharide with an observed rate constant of $\sim 60 \text{ s}^{-1}$ that showed no evidence of any saturable increase over the range $0.5\text{--}20 \mu\text{M}$ heparin, despite tryptophan fluorescence changes and a factor Xa reactivity enhancement indicative of significant conformational activation (Table 1 and Figure 3). This suggested that the binding of the heparin pentasaccharide to native antithrombin had been severely weakened so as to favor the alternative conformational selection mechanism of activation in which the pentasaccharide selectively and rapidly binds to a minor equilibrium fraction of free activated antithrombin, AT^* , with dissociation constant, K_4 , following a rate-limiting transformation of the free native serpin, AT , to the activated state with rate constant, k_{+3} , as given by the scheme (43):

The heparin concentration independent k_{obs} in this case would represent, k_{+3} , the rate constant for conversion of heparin-free native to activated antithrombin, if heparin concentrations were saturating with respect to the activated antithrombin interaction. This is reasonable given that the conformational equilibrium in the absence of heparin favors the native state by at least 100:1 based on a basal antithrombin reactivity with factor Xa that is $\sim 1\%$ that of the heparin-activated reactivity, i.e., $K_3 = k_3/k_{+3} \sim 100$ (32). This would imply that $K_4 = K_{D,obs}/K_3$ is minimally 100-fold less than $K_{D,obs}$ ($\sim 20 \mu\text{M}$) and thus, that the range of heparin concentrations examined were well above saturation.

Effects of heparin activation of antithrombin variants on reactivity with proteases

Conformational activation of antithrombin by the heparin pentasaccharide enhances the reactivity of the serpin with factors Xa and IXa several hundred-fold by promoting favorable

interactions of the RCL-bound proteases with an exosite in sheet C of the serpin (17,18,20,47,48). To determine whether the hotspot heparin binding residues of antithrombin affect the ability of bound heparin to activate antithrombin reactivity with factors Xa and IXa, we performed titrations of the variants with the natural pentasaccharide and monitored increases in factor Xa reactivity (Figure 7). Since factor Xa and IXa reactivities with antithrombin are enhanced by calcium (11), these titrations were done in 20 mM MES buffer, pH 6.0 containing 5 mM CaCl_2 (I 0.023) at 25°C. Compared with the kinetic titrations of select variants performed in I 0.025 sodium phosphate buffer, pH 6 to measure pentasaccharide binding affinity (Figure 3), these titrations required higher heparin pentasaccharide concentrations to achieve saturation since calcium was observed to reduce heparin pentasaccharide affinity for antithrombin. Parallel titrations in the calcium buffer monitored by tryptophan fluorescence increases verified that fractional increases in antithrombin reactivity with factor Xa exactly corresponded to fractional fluorescence increases and thus accurately reflected heparin binding and activation of antithrombin.

Nearly full activation of antithrombin reactivity with factor Xa was observed for all mutants that underwent a significant tryptophan fluorescence enhancement upon heparin pentasaccharide binding despite major losses in heparin affinity. This suggested that these mutants all bound heparin preferentially in the activated state over the native state and thus fully shifted antithrombin into the activated state when heparin was bound, even though heparin affinities were greatly reduced. This was the case for all single mutants as well as the Lys114/Arg129 and Lys125/Arg129 double mutants which showed enhancements in factor Xa reactivity ranging from 73–110% of wild-type and fluorescence enhancements ~50–100% of wild-type upon heparin pentasaccharide binding (Figure 7 & Tables 1 & 7). By contrast, the double Lys114/Lys125 and triple Lys114/Lys125/Arg129 mutant antithrombins showed progressively reduced enhancements of their reactivity with factor Xa of 30 and 4% of wild-type and corresponding minor to undetectable tryptophan fluorescence enhancements upon heparin pentasaccharide binding. This implied a progressive loss in the preferential binding of heparin to the activated state of these mutants and thus a more favorable binding to and stabilization of the native state with low factor Xa reactivity. Notably, the shift in preferential binding of heparin from the activated state to the native state of these mutants was paralleled by only modest changes in overall heparin binding affinity.

To determine whether heparin pentasaccharide binding and activation of antithrombin enhances reactivity with factor IXa to the same extent as with factor Xa, we measured the reactivities of the mutant antithrombins with factor IXa in MES/ Ca^{2+} pH 6 buffer after saturation with heparin pentasaccharide based on the heparin affinities measured for these mutants (Table 7). Surprisingly, the mutations caused much larger losses in heparin activation of antithrombin reactivity with factor IXa than with factor Xa. All single as well as Lys114/Arg129 and Lys125/Arg129 double mutants that showed full or nearly full activation of antithrombin reactivity with factor Xa exhibited marked decreases in reactivity with factor IXa of 21–68% of wild-type. Moreover, the double Lys114/Lys125 and triple mutants that showed large reductions in heparin activation of antithrombin reactivity with factor Xa of 30 and 4% of wild-type showed nearly complete abrogation of activation toward factor IXa, i.e., 3 and 0.3% of wild-type, respectively. Significantly, the basal reactivity of

antithrombin with factors Xa and IXa in the absence of heparin was also affected by the mutations of the heparin binding residues (Table 7). Most interesting, single mutations of Lys125 and Arg129 enhanced antithrombin basal reactivities with both factors Xa and IXa 1.3–2.3-fold and these enhancements were magnified in the double mutant (2.8–3.5-fold). By contrast, mutations of Lys114 alone or with Lys125 and/or Arg129 reduced basal reactivities with factor Xa ~2-fold and with factor IXa 2–10-fold, the single Lys114 mutation having the most pronounced effects. Such effects imply that the mutations perturb the equilibrium between native and activated states of the free serpin, with Lys125 and Arg129 stabilizing the native state and Lys114 stabilizing the activated state (32).

The opposing effects of the hotspot residue mutations on the equilibrium between native and activated states in the absence of heparin was correlated with similar opposing effects of the mutations on thermal stability (Table 1) as well as the fluorescence enhancement accompanying TNS binding to antithrombin. Binding of TNS to wild-type antithrombin produces a marked 30–40 nm blue-shift and large enhancement of TNS fluorescence (41). This fluorescence is quenched 70–80% upon heparin pentasaccharide binding without shifting the fluorescence peak, consistent with TNS binding to a hydrophobic pocket close to the heparin binding site that is perturbed by heparin binding. All antithrombin mutants bound TNS with a blue-shift in the emission maximum indistinguishable from wild-type but with fluorescence enhancements that differed significantly from wild-type (Table 8). In the absence of heparin, Lys125 and Arg129 single mutations greatly enhanced the fluorescence of bound TNS ~3-fold whereas the Lys114 single mutation reduced this fluorescence ~2-fold. Double and triple mutations had intermediate effects, consistent with TNS reporting on an equilibrium mixture of native and activated states of free antithrombin with Lys125/Arg129 mutations amplifying the fraction in activated states with a high fluorescence yield and Lys114 mutations augmenting the fraction in the native state with a low fluorescence yield. Binding of heparin to all mutants produced marked quenches in bound TNS fluorescence of 40–70%, consistent with the efficient quenching of bound TNS fluorescence in both native and activated states of the heparin-bound serpin.

DISCUSSION

The findings of this study provide a clear demonstration that cooperative interactions of three hotspot heparin binding residues of antithrombin are critical for a sequence-specific heparin pentasaccharide to bind and allosterically activate the anticoagulant function of the serpin. Such cooperativity has been suggested from previous findings that mutation of any one of the hotspot residues results in the loss of multiple ionic and nonionic interactions with the pentasaccharide (21–23). The cooperative action of the hotspot residues was most evident from the marked nonadditivity of pentasaccharide binding energy losses when mutations of any one hotspot residue were done in the context of wild-type or mutant forms of the other two residues.

The cooperativity is quantitatively reflected by the substantial coupling energies of the three pairs of hotspot residues and the significant enhancement of these coupling energies in the context of a mutant third residue, a hallmark feature of the three residues acting as a cooperative unit (28,29). The coupling energies revealed that Lys125 or Arg129 interactions

with natural or super pentasaccharides are most strongly coupled to those of Lys114 or to those of each other in wild-type antithrombin and become fully coupled in a mutant third residue context relative to the reciprocal coupling of Lys114 interactions. As a result, Lys125 and Arg129 are completely dependent, and Lys114 mostly dependent, on cooperative interactions with one other hotspot residue to make a positive contribution to overall pentasaccharide binding energy. However, differential effects of the hotspot residue mutations on pentasaccharide affinities for native and activated states discussed below suggest that all three hotspot residues contribute to preferential binding of the pentasaccharide to activated antithrombin even when cooperative interactions with other hotspot residues are eliminated. The modest residual binding energy contribution of Lys114 when cooperative interactions are lost may reflect a residual coupling of Lys114 with other basic residue interactions. Our finding that mutations of Lys114 normalize the binding of antithrombin to natural and super pentasaccharides indeed suggests that Lys114 interactions are coupled with those of Arg47, since Arg47 interacts with the additional sulfate of the super pentasaccharide in the X-ray structure of the antithrombin-pentasaccharide complex (18).

Rapid kinetic studies of pentasaccharide binding to wild-type and variant antithrombins established how the cooperative interactions of the hotspot residues are expressed in the two step binding and activation mechanism. The losses in pentasaccharide binding energy of the variants were thus found to result principally from a reduced ability to drive (k_{+2}) and stabilize (k_{-2}) the induced-fit interaction with the activated antithrombin conformation (as reflected in the conformational equilibrium constant, $K_2 = k_{-2}/k_{+2}$) and much less from reduced interactions with the native conformation (as reflected in the apparent dissociation constant, K_1 , for the native state interaction). Mutating Lys125 or Arg129 singly or together resulted in major 10^2 - 10^4 -fold effects on K_2 , due primarily to increases in k_{-2} , whereas mutation of Lys114 alone caused a massive 10^5 -fold effect on K_2 due to large decreases in k_{+2} and increases in k_{-2} . The conformational activation rate constants could not be determined for mutants in which the Lys114 mutation was combined with mutations of Lys125 or Arg129 or both, since conformational activation occurred through an alternative conformational selection pathway or was progressively suppressed most likely due to Lys125 or Arg129 mutations further increasing the reverse conformational activation rate constant. It was nevertheless clear from the progressive losses in tryptophan fluorescence and factor Xa reactivity enhancements produced by pentasaccharide binding to these mutants that combining the Lys114 mutation with that of Arg129 marginally affected pentasaccharide activation of antithrombin whereas mutating Lys114 together with Lys125 or with both Lys125 and Arg129 caused a progressive loss in pentasaccharide activation of the serpin that culminated in a nearly complete abrogation of activation in the case of the triple mutant. The progressive suppression of pentasaccharide activation of these mutants reflects a gradual loss of the preferential interaction of the pentasaccharide with the activated state and correspondingly more favorable interaction with the native state. The observed pentasaccharide affinity for the triple mutant thus largely reflects an interaction with the native state ($K_{D,obs} \approx K_1$) and hence a $>10^7$ -fold shift of the conformational activation equilibrium (K_2) in favor of the native state.

The more modest effects of the hotspot residue mutations on the initial weak docking interaction of the pentasaccharide with native antithrombin nevertheless suggests an important role of the residues in this interaction. Whereas the affinity of the pentasaccharide for the native state (K_1) was only directly measurable for the three single mutants and one double mutant, this affinity could be inferred for the other double mutants and triple mutant from estimated values of K_2 and measured overall pentasaccharide affinities ($K_{D,obs}$) (Table 6). While single mutations produced relatively minor effects (2–5-fold) on the pentasaccharide interaction with native antithrombin, double mutations resulted in major effects (from ~30–200-fold). The double mutations thus magnified the modest binding energy losses of the single mutations, consistent with the initial docking of the pentasaccharide to native antithrombin involving a weak electrostatic association dependent on the overall constellation of charged residues, rather than on any single residue. However, this trend was altered in the triple mutant which exhibited a lesser ~ 10-fold effect on K_1 than the double mutants. A transition from decreases in native state affinities to increases was in fact clear in the Lys114 double and triple mutants as noted above from the gradual loss in preferential interaction of the pentasaccharide with the activated state and corresponding more favorable interaction with the native state of these mutants (Table 6).

Such gains in native state affinity coupled with losses in activated state affinity suggest that nonproductive modes of binding of the pentasaccharide to the native state compete with the productive binding mode as the hotspot residues are mutated and contribute to favoring the native state over the activated state interaction in the triple mutant. Other basic residues that may promote such nonproductive electrostatic binding of the pentasaccharide include both binding (Lys11, Arg13, Arg47) and nonbinding residues (Arg46, Arg132, Lys133) within or adjacent to the pentasaccharide binding site (Figure 8). The observation that all double mutants retained a substantial or complete ability to be activated by the pentasaccharide suggests that the productive binding mode remains preferred as long as one of the hotspot residues is present, but is ultimately disfavored when all three hotspot residues are mutated. The hotspot residues thus appear to play an important role in electrostatically guiding the pentasaccharide to the productive binding site (49).

The marked cooperativity of the antithrombin hotspot residues in producing an induced-fit high-affinity interaction with the pentasaccharide in the activated state implies that linked structural changes of the three residues mediate this cooperativity (29). X-ray structures of free and pentasaccharide-complexed antithrombin (14,15,18) have revealed that the structural changes which position the hotspot residues for an induced-fit interaction with the pentasaccharide involve the formation of a new P helix in the loop at the N-terminal end of helix D that includes Lys114 and a shift and C-terminal extension of helix D in which Lys125 and Arg129 reside (Figure 1). Studies with truncated pentasaccharides have shown that the rigid nonreducing end trisaccharide, designated DEF, is sufficient to bind and induce the activating structural changes in antithrombin through interactions with the three hotspot residues whereas the flexible reducing end disaccharide, GH, binds only after conformational activation through interactions with Lys114 and other basic residues to lock antithrombin in the activated state (43,46). The cooperativity of the hotspot residues in mediating the induced-fit interaction with the pentasaccharide thus derives from their

interactions with the DEF trisaccharide. Interestingly, a high resolution structure of the antithrombin-pentasaccharide complex reveals that the hotspot residues are part of two key binding pockets in which critical sulfates of the DEF trisaccharide bind (Figure 8). Lys114 and Lys125 along with Lys11, Asn45, Arg47 and Pro12 form one binding pocket for the critical 3-O-sulfate of the central F saccharide and Lys125 and Arg129 along with Thr44 and Asn45 form a second pocket for the critical 6-O-sulfate of the nonreducing end saccharide D (18). The importance of these two sulfate interactions is evident from the findings that elimination of either the 3-O-sulfate or the 6-O-sulfate result in the loss of ~60% of pentasaccharide binding energy (33,43) and that both Lys125 and Arg129 interactions with the pentasaccharide depend on the 6-O-sulfate of saccharide D (21,23). Notably, Lys125 and Asn45 are common to both binding pockets and interact with both of the critical sulfates. Mutation of any one of the hotspot residues would therefore be expected to perturb Lys125 and Asn45 interactions and thereby disturb the induced-fit interactions of the unmutated hotspot residues in both pockets. The juxtaposition of the two binding pockets thus suggest how perturbations of the three hotspot residues are structurally linked and how these residues function cooperatively.

The effects of the hotspot residue mutations on pentasaccharide activation of antithrombin reactivity with factors Xa and IXa surprisingly revealed more marked losses in reactivity with factor IXa than with factor Xa. Such findings are inconsistent with the mutations perturbing a two-state conformational equilibrium between heparin-bound native and activated antithrombin states since this would be expected to produce equivalent fractional changes in the reactivity of heparin-activated antithrombin with factor Xa and factor IXa (32). The results instead support previous proposals of the existence of two activated states (50,51) that make different contributions to the enhancement of factor Xa and factor IXa reactivities. Structural and mutagenesis data suggest that the first activated state involves conformational changes in the heparin binding site that are transmitted to the hydrophobic core whereas the second activated state involves additional changes in the heparin binding site that cause the RCL hinge to be expelled from an interaction with sheet A and result in a major fraction of the tryptophan fluorescence enhancement (50–52). Such findings suggest a greater dependence of the factor IXa reactivity enhancement on both activating conformational changes than the factor Xa reactivity enhancement, although clear correlations between the tryptophan fluorescence enhancements and the differential enhancements in factor Xa and IXa reactivities are difficult to discern.

Interestingly, the basal reactivities of the antithrombin mutants with factor Xa and factor IXa, but not with thrombin, were significantly affected by the hotspot residue mutations. These effects were correlated with changes in thermal stability as well as changes in the fluorescence of the bound probe, TNS, which is thought to interact close to the heparin binding site of antithrombin (51). Such findings suggest that the three hotspot residues function to differentially stabilize native and activated states of free antithrombin, with Lys114 stabilizing the activated state(s) and both Lys125 and Arg129 stabilizing the native state. This markedly contrasts with the massive stabilization of the activated state by the cooperative action of all three residues when the pentasaccharide is bound.

CONCLUSIONS

Our findings reveal the molecular basis for the hotspot binding of three basic residues of antithrombin to the heparin pentasaccharide activator of the serpin and demonstrate their critical role in mediating allosteric activation of antithrombin by heparin. The overriding contributions of the three hotspot residues to pentasaccharide binding energy arise from strong coupling interactions between pairs of residues that depend on the state of the third residue, indicative of the three residues acting as a cooperative unit. Rapid kinetic studies demonstrate that the three residues act both to electrostatically guide the nonreducing end trisaccharide of the pentasaccharide to a specific productive binding site on native antithrombin and to cooperatively induce linked structural changes in the heparin binding site that result in a complementary high-affinity interaction with the full pentasaccharide and the transmission of global structural changes that activate the serpin. A high resolution structure of the antithrombin-pentasaccharide complex suggests that the cooperativity of the hotspot residues is mediated by linked structural changes of two adjacent binding pockets comprised of pairs of hotspot residues that interdependently interact with critical sulfates of the pentasaccharide. The cooperative interactions of the hotspot residues in mediating allosteric activation of antithrombin by heparin has important implications for understanding the evolution of this unique allosterically activated clade C serpin (53) as well as for the design of heparin mimetic activators of antithrombin (54).

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ABBREVIATIONS:

RCL	reactive center loop
TNS	2-p-toluidinylnaphthalene-6-sulfonate
MES	2-(N-morpholino)ethanesulfonic acid

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Figure 1: Hotspot binding residues in the heparin binding site of antithrombin.

The helix D region of the heparin binding site in antithrombin is shown in free (cyan, pdb: 1EO3) and heparin pentasaccharide-complexed (green, pdb: 3KCG) structures of antithrombin after aligning the 238 residue large fragments of the serpin whose structures do not change in the two states (25). The pentasaccharide (designated DEFGH) is shown in red stick with two critical sulfates indicated and the sidechains of the hotspot residues, Lys114, Lys125 and Arg129, are depicted in stick. The structural changes that produce an induced-fit

interaction with the pentasaccharide include the formation of helix P in the N-terminal unstructured loop of helix D and the tilting and C-terminal extension of helix D.

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Figure 2: SDS-PAGE of wild-type and mutant antithrombins and their complexes with thrombin
Purified wild-type (WT) and variant antithrombins with mutations of the three hotspot heparin binding residues indicated as in Table 1 were electrophoresed alone and after reaction with a molar excess of thrombin (Ila) under reducing conditions as described in “Materials and Methods”. Paired lanes in gels on the left and right show each of the antithrombin mutants without and with added thrombin. The two lanes in the far right of the left-hand gel are pure thrombin and standard molecular markers (kDa). The positions of free antithrombin, thrombin and antithrombin-thrombin complexes are indicated. Low molecular weight bands migrating at the bottom of each lane represent the thrombin A chain and C-terminal peptide of antithrombin released upon formation of complexes.

Figure 3: Kinetic titrations of variant antithrombin interactions with the natural pentasaccharide

A, Binding curves for the interaction of the natural pentasaccharide with the indicated variant antithrombins were measured from the saturable increase in the apparent second order rate constant (k_a) for antithrombin inhibition of factor Xa as a function of increasing pentasaccharide concentration. Values of k_a were measured by reacting antithrombin and pentasaccharide with factor Xa under pseudo-first order conditions in sodium phosphate buffer, pH 6, I 0.025 at 25°C for different fixed time intervals and quantitating the extent of factor Xa inhibition as described in Materials and Methods. Solid lines are fits of binding curves by the quadratic equilibrium binding equation 1 from which values of $K_{D,obs}$ were derived. **B,** The binding curves for the two variants with the smallest enhancements in k_a are shown on an expanded scale.

Figure 4: Ionic strength dependence of antithrombin-pentasaccharide interactions

The logarithm of observed dissociation constants ($K_{D,obs}$) for the binding of the natural pentasaccharide (H5, panel A) or the super pentasaccharide (H5*, panel B) to wild-type and variant antithrombins is plotted as a function of the logarithm of the sodium ion concentration. Antithrombin variants with single, double or triple mutations of Lys114, Lys125 and Arg129 residues to Met are designated in that order by the single letter amino acid code with wild-type given by KKR. $K_{D,obs}$ values were measured by monitoring tryptophan fluorescence changes accompanying the titration of pentasaccharides into solutions of antithrombin at 25°C in sodium phosphate buffer containing varying concentrations of NaCl adjusted to pH 6. Binding of the natural pentasaccharide to MMR and MMM variants which yielded insignificant fluorescence changes was monitored by titrating changes in the apparent rate constant for factor Xa inactivation as shown in Figure 3. Further details are provided in Materials and Methods. Solid lines are linear regression fits of the data based on equation 4.

Figure 5: Double mutant cycles for analyzing the additivity of mutational effects on antithrombin-pentasaccharide interactions

Left panel, A double mutant cycle depicting the two possible sequences of mutating antithrombin Lys114 and Lys125 residues to Met. Antithrombin variants are designated by the single letter amino acid code with wild-type indicated by K₁₁₄K₁₂₅R₁₂₉. The extent to which these residues interact in binding the pentasaccharide is indicated from the difference in the binding free energy change when either residue is mutated in the context of a wild-type ($G^\circ_{\text{K114} \rightarrow \text{M}, \text{K125}}$, $G^\circ_{\text{K114}, \text{K125} \rightarrow \text{M}}$) versus a mutated paired residue ($G^\circ_{\text{K114} \rightarrow \text{M}, \text{M125}}$, $G^\circ_{\text{M114}, \text{K125} \rightarrow \text{M}}$). This difference represents the coupling free energy. *Right panel,* The mutation of Lys114, Lys125 and Arg129 residues in all possible sequences involves six double mutant cycles defined by the faces of a cube wherein mutations of a pair of residues are considered in the context of a wild-type or a mutant third residue. The residue numbers are omitted but the designations are the same as in the left panel.

Figure 6: Rapid kinetics of heparin pentasaccharide binding to antithrombin variants

A, The dependence of the observed pseudo-first order rate constant for pentasaccharide binding to antithrombin (k_{obs}) on the pentasaccharide concentration is shown for wild-type (KKR, ●) and variant antithrombins, KKM (▲), KMR (◆), KMM (▼), MKM (▷) and MKR (■). Values of k_{obs} were measured by monitoring tryptophan fluorescence changes accompanying the binding of the natural pentasaccharide to antithrombin with a stopped-flow fluorometer under pseudo-first order conditions in 0.04 M sodium phosphate buffer, pH 6 at 25°C as described in Materials and Methods. Wild-type and variants are designated as in previous figures. Solid lines are fits of data by the hyperbolic equation 5 in the text. Each point represents the average of at least five progress curves with the error bars indicating $\pm$ S.E. **B,** Data for MKM and MKR antithrombin variants shown on an expanded scale. The horizontal line through the MKM variant data indicates the average of values measured over all pentasaccharide concentrations.

Figure 7: Effects of hotspot residue mutations on pentasaccharide activation of antithrombin reactivity with factor Xa

Titration of the enhancement in the apparent second order association rate constant (k_a) for antithrombin inhibition of factor Xa as a function of increasing concentrations of the natural pentasaccharide are shown for wild-type (KKR, ●) and variant antithrombins, KKM (□), KMR (▲), KMM (△) in *panel A* and for variant antithrombins, MKR (○), MKM (■), MMR (◆) and MMM (◇) in *panel B*. Values of k_a were measured from reactions of antithrombin with factor Xa in the presence of pentasaccharide under pseudo-first order conditions in 20 mM MES, 5 mM CaCl_2 buffer, pH 6 at 25°C as described in Materials and Methods. The apparent k_a was calculated by dividing the observed pseudo-first order rate constant by the antithrombin concentration. Solid lines are fits of data by the quadratic equation 1 for equilibrium binding of the pentasaccharide to antithrombin. The dashed line in panel B represents the fitted maximal wild-type enhancement in k_a from panel A.

Figure 8: The hotspot residues form two structurally linked binding pockets that interact with critical sulfates of the heparin pentasaccharide

Surface representation of the heparin binding site of the antithrombin-pentasaccharide complex (pdb: 3KCG) reveals two adjoining binding pockets formed by the hotspot residues (blue) for the critical 6-O-sulfate of pentasaccharide residue D and the critical 3-O-sulfate of saccharide F of the nonreducing end DEF trisaccharide that mediates conformational activation of antithrombin. Additional residues that line these pockets and contribute to the linked interactions are depicted in green. The participation of Lys125 as well as Asn45 in both binding pocket interactions suggests a structural basis for the cooperative interactions of the hotspot residues with the pentasaccharide. Less critical basic residues that contribute to binding the GH reducing end disaccharide following conformational activation by the DEF trisaccharide are shown in light blue. Other nonbinding basic residues on the periphery

of the binding site that may contribute to nonproductive binding of the pentasaccharide to mutant antithrombins are colored cyan.

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Table 1:
Thrombin reactivity, heat stability, and heparin-induced tryptophan fluorescence changes of variant antithrombins

Association rate constants (k_a) for antithrombin inhibition of thrombin in the absence of heparin were measured under pseudo-first order conditions in 0.025 M sodium phosphate buffer, pH 6.0 at 25°C as described in Materials and Methods. Values represent averages $\pm$ S.E. from at least three independent measurements. Average melting temperatures $\pm$ range of antithrombin variants were measured from duplicate thermal titrations monitored by tryptophan fluorescence changes in 0.15 M sodium phosphate buffer, pH 7.4 as described in Materials and Methods. Average maximal tryptophan fluorescence enhancements $\pm$ S.E. induced in antithrombin by the binding of the natural (H5) or super (H5*) heparin pentasaccharide were measured by equilibrium binding titrations in 20 mM NaPi buffer, pH 6 over a range of NaCl concentrations at 25°C after correction of the initial fluorescence for nonfunctional protein.

Antithrombin variant	k_a (M ⁻¹ s ⁻¹)	T_m (°C)	F_{max}/F_o H5	F_{max}/F_o H5*
Wild-type (KKR)	$1.1 \pm 0.1 \times 10^{3a}$	56.2 ± 0.3	0.36 ± 0.01	0.39 ± 0.01
K114M (MKR)	$9.5 \pm 0.4 \times 10^2$	59.1 ± 0.2	0.32 ± 0.01	0.31 ± 0.01
K125M (KMR)	$8.0 \pm 0.3 \times 10^2$	54.7 ± 0.3	0.35 ± 0.01	0.41 ± 0.01
R129M (KKM)	$1.0 \pm 0.1 \times 10^3$	55.7 ± 0.1	0.36 ± 0.01	0.34 ± 0.01
K114M/K125M (MMR)	$1.5 \pm 0.4 \times 10^3$	58.1 ± 0.2	0.06 ± 0.01	---
K114M/R129M (MKM)	$9.3 \pm 0.7 \times 10^2$	58.0 ± 0.3	0.28 ± 0.01	0.15 ± 0.01
K125M/R129M (KMM)	$6.9 \pm 0.5 \times 10^2$	52.9 ± 0.6	0.17 ± 0.02	0.28 ± 0.03
K114M/K125M/R129M (MMM)	$1.1 \pm 0.2 \times 10^3$	57.0 ± 0.4	0^b	---

^aValue indistinguishable from that measured for wild-type antithrombin without a His-tag (43).

^bNo fluorescence change detectable up to 30 μ M H5

Table 2:
Ionic and nonionic contributions to the binding of natural and super pentasaccharides to antithrombin variants at pH 6.0, 25°C

The number of ionic interactions (Z) and logarithm of the dissociation constant for nonionic interactions ($\log K'_{AT,H5}$ or $\log K'_{AT,H5^*}$) for the binding of the natural pentasaccharide (H5) or the super pentasaccharide (H5*) to wild-type and variant antithrombins were determined from the slopes and intercepts of linear regression fits of the dependence of the logarithm of the observed dissociation constant ($\log K_{D,obs}$) on $\log [Na^+]$ shown in Figure 4 based on equation 4, assuming a value of 0.8 for Ψ . Errors represent $\pm$ S.E. of fitted parameters. ND indicates values not determined.

Antithrombin	Z_{H5}	Z_{H5^*}	$\log K'_{AT,H5}$	Z_{H5^*}	AZ_{H5^*}	$\log K'_{AT,H5^*}$
KKR (WT)	6.2 $\pm$ 0.3	---	-5.8 $\pm$ 0.1 (1)	7.1 $\pm$ 1.7	---	-7.7 $\pm$ 0.1 (1)
MKR	3.2 $\pm$ 0.1	3.0	-2.6 $\pm$ 0.2 (0.45)	3.7 $\pm$ 0.3	3.4	-1.6 $\pm$ 0.4 (0.21)
KMR	4.1 $\pm$ 0.2	2.1	-4.4 $\pm$ 0.1 (0.76)	4.9 $\pm$ 0.3	2.2	-6.1 $\pm$ 0.1 (0.79)
KKM	4.4 $\pm$ 0.2	1.8	-4.8 $\pm$ 0.1 (0.83)	5.5 $\pm$ 0.4	1.6	-6.3 $\pm$ 0.1 (0.82)
MMR	2.5 $\pm$ 0.3	3.7	-1.5 $\pm$ 0.4 (0.26)	ND	---	ND
MKM	2.3 $\pm$ 0.1	3.9	-2.1 $\pm$ 0.3 (0.36)	1.7 $\pm$ 0.3	5.4	-3.0 $\pm$ 0.5 (0.39)
KMM	4.4 ^a	1.8	-2.0 ^b (0.34)	5.4 $\pm$ 0.4	1.7	-3.8 $\pm$ 0.2 (0.49)
MMM	2.4 $\pm$ 0.1	3.8	-2.3 $\pm$ 0.1 (0.401)	ND	---	ND

^a Z assumed to be one ionic interaction less than the super pentasaccharide interaction based on the differential affinities of the single KMR and KKM mutants for natural and super pentasaccharides measured over a range of ionic strengths that reflect one less ionic interaction and the parallel differential affinities of the KMM mutant for the two pentasaccharides measured at I 0.025.

^b Calculated from equation 4 based on $K_{D,obs}$ measured at I 0.025 and the assumed value of Z .

Table 3:
Dissociation constants and binding free energy changes for the binding of natural and super pentasaccharides to variant antithrombins at I 0.025, pH 6, 25°C

Dissociation constants for antithrombin interactions with pentasaccharides were measured directly by equilibrium or kinetic titrations or calculated from binding constants measured by titrations at higher ionic strengths based on equation 4 as described in Materials and Methods.

Antithrombin	$K_{AT,H5}$	G°_{bind}	$G^{\circ}_{bind}{}^a$	$K_{AT,H5}^*$	G°_{bind}	$G^{\circ}_{bind}{}^a$
	M	kcal/mole	kcal/mole	M	kcal/mole	kcal/mole
KKR(WT)	1.0×10^{-14}	-19.0	0	7.3×10^{-18}	-23.3	0
MKR	$1.5 \pm 0.1 \times 10^{-7b}$	-9.3	+9.7 (0.51)	$3.0 \pm 0.2 \times 10^{-7b}$	-8.9	+14.4 (0.62)
KMR	1.5×10^{-10}	-13.4	+5.6 (0.29)	2.3×10^{-13}	-17.2	+6.1 (0.26)
KKM	2.5×10^{-11}	-14.4	+4.6 (0.24)	2.2×10^{-14}	-18.6	+4.7 (0.20)
MMR	1.2×10^{-5c}	-6.7	+12.3 (0.65)	1×10^{-5d}	-6.8	+16.5 (0.71)
MKM	$6.6 \pm 0.1 \times 10^{-6b}$ (6.3×10^{-6}) ^c	-7.0	+12.0 (0.63)	$6.8 \pm 0.6 \times 10^{-6b}$	-7.0	+16.3 (0.70)
KMM	$1.4 \pm 0.1 \times 10$ (1.5×10^{-8}) ^o	-10.7	+8.3 (0.44)	1.1×10^{-11}	-14.9	+8.4 (0.36)
MMM	3.3×10^{-6c}	-7.4	+11.6 (0.61)	3×10^{-6d}	-7.5	+15.8 (0.68)

^a $G^{\circ}_{bind} = -1.36 \log K_{D,WT}/K_{D,mut} = G^{\circ}_{bind,mut} - G^{\circ}_{bind,WT}$; values in parentheses express G°_{bind} relative to $G^{\circ}_{bind,WT}$

^b Directly measured at I 0.025; average from 2–4 titrations $\pm$ S.E.

^c Measured by kinetic titrations monitored from increases in k_a for the inhibition of factor Xa produced by the saccharide

^d Assumed to be equal to the value measured for the natural pentasaccharide

Table 4:
Natural and super pentasaccharide binding free energy changes due to mutation of
antithrombin hotspot residues in wild-type and mutant contexts

Pentasaccharide binding free energy changes resulting from mutations of individual hotspot binding residues in different contexts were determined from double mutant cycles as described in the text.

	$G^{\circ}_{\text{bind,H5}}$	$G^{\circ}_{\text{bind,H5}^*}$
Lys114→Met	kcal/mole	kcal/mole
KKR→MKR	+9.7	+14.4
KMR→MMR	+6.7	+10.4
KKM→MKM	+7.4	+11.6
KMM→MMM	+3.3	+7.4
Lys125→Met		
KKR→KMR	+5.6	+6.1
MKR→MMR	+2.6	+2.1
KKM→KMM	+3.7	+3.7
MKM→MMM	-0.4	-0.5
Arg129→Met		
KKR→KKM	+4.6	+4.7
MKR→MKM	+2.3	+1.9
KMR→KMM	+2.7	+2.3
MMR→MMM	-0.7	-0.7

Table 5:
Coupling free energies of pairs of antithrombin hotspot binding residues in wild-type and mutant contexts

Coupling free energies were determined from the data in Table 4 based on double mutant cycles as described in the text.

	$G^{\circ}_{\text{coupling,WT,H5}}$	$G^{\circ}_{\text{coupling,mut,H5}}$	$G^{\circ}_{\text{coupling,WT,H5}^*}$	$G^{\circ}_{\text{coupling,mut,H5}^*}$
Lys114/Lys125	-3.0	-4.1	-4.0	-4.2
Lys114/Arg129	-2.3	-3.4	-2.8	-3.0
Lys125/Arg129	-1.9	-3.0	-2.4	-2.6

Table 6:
Kinetic parameters for binding of the natural heparin pentasaccharide to wild-type and variant antithrombins at I 0.04, pH 6.0, 25°C.

Kinetic parameters $\pm$ S.E. were derived from computer fits of the dependence of observed pseudo-first order rate constants for heparin binding to antithrombin variants on heparin concentration according to equation 5 of the text, as shown in figure 6 and described in materials and Methods.

AT mutant	K_1 (μ M)	k_{+2} s ⁻¹	k_{-2} s ⁻¹	$K_{D,obs}$ (μ M) ^a	$1/K_2$ ^b
KKR (WT)	0.9 $\pm$ 0.2	1140 $\pm$ 110	—	1.5×10^{-7}	6×10^6
MKR	5 $\pm$ 3	17 $\pm$ 2	23 $\pm$ 2 ^c	0.56	8 ^c
KMR	3.7 $\pm$ 0.7	550 $\pm$ 40	—	8.6×10^{-4}	4300
KKM	1.6 $\pm$ 0.3	1020 $\pm$ 120	—	1.6×10^{-4}	10000
MMR	$\sim$ 70 ^e	—	—	45	$\sim$ 0.5 ^d
MKM	$\sim$ 200 ^e	—	—	18	$\sim$ 10 ^d
KMM	29 $\pm$ 15	690 $\pm$ 240	74 $\pm$ 7 ^c	0.07	$\sim$ 400 ^c
MMM	$\sim$ 9 ^e	—	—	8.4	$\sim$ 0.03 ^d

^a Measured directly at I 0.04 or calculated from the ionic strength dependence of $K_{D,obs}$

^b $1/K_2 = (K_1/K_{D,obs}) - 1$

^c Deviations from values of $1/K_2$ calculated from the ratio, k_{+2}/k_{-2} , suggest that k_{-2} is overestimated due to a contribution of the conformational selection pathway of pentasaccharide binding and activation (21,23).

^d Estimated based on factor Xa reactivity enhancements

^e Calculated from measured values of $K_{D,obs}$ and estimated values of K_2 based on $K_1 = K_{D,obs} \times (1 + K_2)/K_2$

Table 7:
Association rate constants for reactions of free (k_{-H}) and heparin-complexed (k_H) antithrombin variants with factor Xa and factor IXa

Reactions of antithrombin variants with proteases were performed in 20 mM MES, 5 mM CaCl₂, 0.1 % PEG 8000, pH 6.0 at 25°C under pseudo-first order conditions as described in Materials and Methods. Second order rate constants were calculated from pseudo-first order rate constants by dividing by the antithrombin or antithrombin-pentasaccharide complex concentration based on dissociation constants measured for the latter interaction. Errors represent standard errors of at least three determinations. Values in parentheses represent fractional wild-type values.

Antithrombin	k _{-H} FXa (M ⁻¹ s ⁻¹)	k _H FXa (M ⁻¹ s ⁻¹)	k _{-H} FIXa (M ⁻¹ s ⁻¹)	k _H FIXa (M ⁻¹ s ⁻¹)
KKR (WT)	5.7±0.4 × 10 ² (1)	7.7±0.4 × 10 ⁴ (1)	5.4±0.4 × 10 ¹ (1)	1.0±0.2 × 10 ⁴ (1)
MKR	2.6±0.3 × 10 ² (0.46)	6.4±0.3 × 10 ⁴ (0.83)	6±1×10 ⁰ (0.11)	2.1±0.1 × 10 ³ (0.21)
KMR	1.2±0.1 × 10 ³ (2.1)	8.1±0.3 × 10 ⁴ (1.1)	9.6±0.6 × 10 ¹ (1.8)	6.8±0.8 × 10 ³ (0.68)
KKM	1.3±0.1 × 10 ³ (2.3)	7.6±0.3 × 10 ⁴ (0.99)	6.9±0.4 × 10 ¹ (1.3)	6.2±0.3 × 10 ³ (0.62)
MMR	3.4±0.4 × 10 ² (0.60)	2.3±0.3 × 10 ⁴ (0.30)	8±2×10 ⁰ (0.15)	3.0±0.1 × 10 ² (0.03)
MKM	3.5±0.5 × 10 ² (0.61)	8.5±0.4 × 10 ⁴ (1.1)	1.3±0.2×10 ¹ (0.24)	2.1±0.1 × 10 ³ (0.21)
KMM	2.0±0.1 × 10 ³ (3.5)	5.6±0.4 × 10 ⁴ (0.73)	1.5±0.2 × 10 ² (2.8)	3.6±0.2 × 10 ³ (0.36)
MMM	3.0±0.4 × 10 ² (0–53)	3.1±0.3 × 10 ³ (0.04)	2.3±0.2 × 10 ¹ (0.43)	3.4±0.2 × 10 ¹ (0.003)

Table 8:
Relative fluorescence of TNS bound to antithrombin variants in the absence and presence of saturating heparin pentasaccharide.

Fluorescence spectra of 10 μM TNS were measured in 0.025 NaPi buffer pH 6 at 25°C before and after adding 0.5 μM antithrombin variant and after titrating in saturating heparin pentasaccharide as described in Materials and Methods. Spectra of TNS bound to antithrombin with and without complexed pentasaccharide were determined by subtraction of the free TNS spectrum and the peak fluorescence intensity of bound TNS in the absence ($F_{\text{TNS,b,-H5}}$) or presence ($F_{\text{TNS,b,+H5}}$) of saturating heparin pentasaccharide expressed relative to the peak intensity of free TNS ($F_{\text{TNS,f}}$). Each set of spectra were measured at least two times and average relative fluorescence intensities $\pm$ S.E. reported.

Antithrombin	$F_{\text{TNS,b,-H5}}/F_{\text{TNS,f}}^a$	$F_{\text{TNS,b,+H5}}/F_{\text{TNS,f}}$	$F_{\text{TNS,b,+H5}}/F_{\text{TNS,b,-H5}}$
KKR (WT)	1.23 $\pm$ 0.03	0.33 $\pm$ 0.03	0.27 $\pm$ 0.02
MKR	0.76 $\pm$ 0.03	0.42 $\pm$ 0.01	0.56 $\pm$ 0.02
KMR	3.20 $\pm$ 0.01	1.54 $\pm$ 0.01	0.48 $\pm$ 0.01
KKM	2.74 $\pm$ 0.13	1.20 ^b	0.42 ^b
MMR	1.62 $\pm$ 0.01	0.82 $\pm$ 0.03	0.51 $\pm$ 0.02
MKM	0.56 $\pm$ 0.02	0.32 $\pm$ 0.02	0.58 $\pm$ 0.02
KMM	3.53 $\pm$ 0.08	1.15 $\pm$ 0.12	0.32 $\pm$ 0.03
MMM	2.13 $\pm$ 0.07	1.10 $\pm$ 0.08	0.52 $\pm$ 0.02

^aSince TNS binds weakly to antithrombin ($K_d > 10 \mu\text{M}$), the bound TNS concentration at 10 μM free TNS will be much less than the antithrombin concentration (0.5 μM) and therefore the reported ratios greatly underestimate the enhancement of TNS fluorescence upon binding.

^bValue from a single titration.