THE SIGNATURE 3-O-SULFO GROUP OF THE ANTICOAGULANT HEPARIN SEQUENCE IS CRITICAL FOR HEPARIN BINDING TO ANTITHROMBIN BUT IS NOT REQUIRED FOR ALLOSTERIC ACTIVATION

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Running head: Role of Heparin 3-O-Sulfate in Antithrombin Activation
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Heparin and heparan sulfate glycosaminoglycans allosterically activate the serpin, antithrombin, by binding through a specific pentasaccharide sequence containing a critical 3-O-sulfo group. To elucidate the role of the 3-O-sulfo group in the activation mechanism, we compared the effects of deleting the 3-O-sulfo group or mutating the Lys114 binding partner of this group on antithrombin-pentasaccharide interactions by equilibrium binding and rapid kinetic analyses. Binding studies over a wide range of ionic strength and pH showed that loss of the 3-O-sulfo group caused a massive ~60% loss in binding energy for the antithrombin-pentasaccharide interaction due to the disruption of a cooperative network of ionic and nonionic interactions. Despite this affinity loss, the 3-O-desulfonated pentasaccharide retained the ability to induce tryptophan fluorescence changes and enhanced factor Xa reactivity in antithrombin indicative of normal conformational activation. Rapid kinetic studies showed that loss of the 3-O-sulfo group affected both the ability of the pentasaccharide to recognize native antithrombin as well as to preferentially bind and stabilize activated antithrombin. By contrast, mutation of Lys114 solely affected the preferential interaction of the pentasaccharide with activated antithrombin. These findings demonstrate that the 3-O-sulfo group functions as a key determinant of heparin pentasaccharide activation of antithrombin both by contributing to the Lys114-independent recognition of native antithrombin and by triggering a Lys114-dependent induced-fit interaction with activated antithrombin that locks the serpin in the activated state.

Antithrombin, a member of the serpin superfamily of protein protease inhibitors, is the principal physiologic regulator of blood coagulation proteases in vertebrates (1,2). Deficiencies of this blood plasma protein thus increase the risk of thrombotic disease (3) and complete deficiency appears to be incompatible with life (4). Antithrombin regulates the activity of its major target proteases, thrombin, factor Xa and factor IXa, by inactivation of the enzymes through a branched pathway suicide substrate mechanism of inhibition that is characteristic of serpin-protease reactions (5). In this mechanism, the protease initially recognizes an exposed reactive loop of the serpin as a normal substrate and proceeds to cleave the loop and form the usual acyl-intermediate. However, once this cleavage has occurred, the serpin is induced to undergo a massive conformational change in which the N-terminal part of the reactive loop inserts into the major β-sheet of the serpin, causing the acyl-linked protease to be dragged to the opposite end of the protein inhibitor and inactivated through conformational distortion (6-8).

In contrast to the rapid rates at which many serpins inactivate their target proteases, antithrombin inhibits coagulation proteases at slow nonphysiologic rates. However, these inhibition rates increase up to several thousand-fold in the presence of the sulfated glycosaminoglycans, heparin and heparan sulfate, which thereby act as efficient anticoagulants (9). The accelerating effects of heparin and heparan sulfate on antithrombin-protease reactions is dependent on the binding of a sequence-specific pentasaccharide to the serpin (10,11), present in about one-third of naturally occurring heparin chains and in a much smaller fraction of heparan sulfate chains (12). A characteristic structural marker of the specific
antithrombin-binding pentasaccharide region is the 3-O-sulfonated central glucosamine residue (Fig. 1) which is absent or rare in other parts of the heparin molecule (10). The X-ray structure of the antithrombin-pentasaccharide complex and mutagenesis studies have shown that antithrombin basic residues in the N-terminal region, helix A, helix D and the loop preceding helix D, form a positively charged site to which the negatively charged pentasaccharide binds (13). Lys114, Lys125 and Arg129 are the most important of these residues, with Lys114, the binding partner of the 3-O-sulfo group, contributing the greatest binding energy (9,14).

In this work, we have studied the interaction of a variant pentasaccharide lacking the 3-O-sulfo group with antithrombin. This variant has been previously reported to poorly bind antithrombin and to have greatly reduced anticoagulant activity, suggesting an essential role of the 3-O-sulfo group in binding and activating antithrombin (15-18). However, quantitative studies were done at physiologic ionic strength and pH where binding to antithrombin was extremely weak and the extent of inhibitor activation was difficult to quantify. We show, by analyzing pentasaccharide binding to and activation of α- and β-glycoforms of antithrombin over a wide range of ionic strength and pH, that deletion of the 3-O-sulfo group results in tremendous ~10^4-10^5-fold losses in affinity for antithrombin under physiologic conditions due to the disruption of a cooperative network of ionic and nonionic interactions that stabilize the complex. Despite these large losses in affinity and in contrast to past reports (16), loss of the 3-O-sulfo group does not affect the ability of the pentasaccharide to induce conformational activation of antithrombin. Rapid kinetic studies of the variant pentasaccharide interaction with antithrombin reveal marked effects of 3-O-desulfonation on both initial binding and subsequent conformational activation steps. These effects contrast with the effects of mutating the Lys114 binding partner of the 3-O-sulfo group which exclusively involve the second conformational activation step (14). Together, our findings show that the 3-O-sulfo group is of greater quantitative importance for the antithrombin-pentasaccharide interaction than any other pentasaccharide sulfo group or mutated antithrombin investigated so far due to its dual role in recognizing the native antithrombin conformation as well as in preferentially binding and stabilizing the activated antithrombin conformation. In the latter role, our results suggest that the 3-O-sulfo group must engage Lys114 to position pentasaccharide and antithrombin residues for an induced-fit interaction that enhances affinity and locks antithrombin in the activated state.

**EXPERIMENTAL PROCEDURES**

**Antithrombin-** N135Q and K114M/N135Q recombinant antithrombin variants that lacked glycosylation at Asn135, and thereby mimicked the natural β-form of plasma antithrombin, were expressed in BHK cells and purified as previously reported (14). Fully glycosylated α-antithrombin was isolated from human plasma as described (19). Protein concentrations were determined from the 280 nm absorbance using a molar absorption coefficient of 37,700 M^-1 cm^-1 (20).

**Proteases and saccharides-** The synthetic pentasaccharide, DEFGH, corresponding to the antithrombin binding sequence in high-affinity heparin chains and a variant pentasaccharide lacking the 3-O-sulfo group of the central saccharide F were generously provided by Sanofi-Aventis (Toulouse, France) (Fig. 1). The concentration of the natural pentasaccharide was based on stoichiometric titrations of antithrombin with the saccharide at inhibitor concentrations exceeding K_D, as described previously (11,19). These concentrations agreed well with concentrations determined by weight and the molecular mass of the saccharides. The concentration of the 3-O-desulfonated pentasaccharide was determined based on the weight and molecular mass of the saccharide. Human α-thrombin was a gift from Dr. John Fenton (formerly of the New York State Department of Health). Human factor Xa (mostly alpha form) was purchased from Enzyme Research (South Bend, IN). Concentrations of proteases were assessed from their activities in standard assays with peptidyl-p-nitroanilide chromogenic substrates and were based on calibration of these assays with active-site titrated enzymes (21).

**Experimental Conditions-** All experiments were carried out at 25 °C. Studies of the 3-O-
desulfonated and natural pentasaccharide interactions with N135Q antithrombin and plasma antithrombin or of the natural pentasaccharide interaction with K114M/N135Q antithrombin were done in buffers of 10-20 mM sodium phosphate, 0.1 mM EDTA, 0.1% (w/v) polyethylene glycol 8000, adjusted to pH 6.0 or pH 7.4. The ionic strength of the 20 mM sodium phosphate buffer is 0.025 at pH 6 whereas 10-20 mM sodium phosphate buffers at pH 7.4 correspond to ionic strengths of 0.025-0.05. NaCl was added to achieve higher ionic strengths. For experiments in pH 6 buffer, antithrombin was extensively diluted from a pH 7.4 buffer just prior to the experiment to minimize losses in activity due to reduced stability at the lower pH.

**Affinities of antithrombin-pentasaccharide interactions** - Dissociation equilibrium constants for the binding of the different pentasaccharides to N135Q, K114M/N135Q and plasma antithrombins were measured by titrations monitored by the enhancement of the intrinsic protein fluorescence accompanying the binding, as in past studies (11,14,19). Affinities of the natural and variant pentasaccharides for recombinant and plasma antithrombins were determined at pH 6.0 or pH 7.4 with antithrombin concentrations ranging from 50-2000 nM. Titrations were computer fit by nonlinear least-squares analysis to the quadratic binding equation:

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

(Eq. 1)

where \(\Delta F_{\text{obs}}/F_0\) and \(\Delta F_{\text{max}}/F_0\) are the observed and maximal changes in fluorescence relative to the initial fluorescence, respectively, \([\text{AT}]_o\) and \([\text{H}]_o\) are the total antithrombin and heparin concentrations, respectively, \(K_{D,\text{obs}}\) is the observed dissociation constant and \(n\) is the binding stoichiometry. The binding stoichiometry was assumed to be 1 and \(K_{D,\text{obs}}\) and \(\Delta F_{\text{max}}/F_0\) were fitted as parameters. Corrections were made for nonbinding protein in the recombinant antithrombin preparations based on measured stoichiometries of thrombin inhibition as in past studies (14).

**Kinetics of Pentasaccharide Binding** - The kinetics of pentasaccharide binding to antithrombin were analyzed under pseudo-first order conditions as in past studies (11,14) by monitoring the increase in protein fluorescence that accompanies binding in an Applied Biophysics SX-17MV stopped-flow instrument. Experiments analyzing the binding of the natural and desulfonated pentasaccharides to N135Q antithrombin or of the natural pentasaccharide to K114M/N135Q antithrombin were done at I 0.04, pH 6.0. Pentasaccharide concentrations were at least 5-fold higher than antithrombin concentrations. Progress curves were fit by a single exponential function to give the observed pseudo-first order rate constant, \(k_{\text{obs}}\). Multiple curves were averaged to improve signal/noise at subsaturating saccharide concentrations and reported \(k_{\text{obs}}\) values reflect global average values from at least 20 reaction traces. For binding of the desulfonated pentasaccharide to antithrombin, fluorescence amplitudes were confirmed to increase nearly proportionally with saccharide concentration over the range examined, in accordance with the measured \(K_D\) for the interaction. Simulations of the kinetics of heparin binding to antithrombin based on kinetic parameters derived from fitting progress curve data to the general two-step binding mechanism were done using Global Kinetic Explorer software (Kintek corporation, Austin, TX) (22).

**Protease Inhibition** - Stoichiometries of uncatalyzed and pentasaccharide-catalyzed reactions of N135Q, K114M/N135Q and plasma antithrombins with thrombin or factor Xa were determined as described previously (19). A fixed concentration of protease (100-1000 nM) was incubated with increasing molar ratios of inhibitor to enzyme of 1-2 in 50-100 µl of I 0.025, pH 6 or I 0.15, pH 7.4 sodium phosphate buffers for times sufficient to achieve complete reaction based on measured reaction rate constants. When present, normal or variant pentasaccharides were added at a fixed level to yield comparable extents of saturation of antithrombin based on measured \(K_D\) values such that the pentasaccharide-accelerated reaction was dominant. Residual enzyme activity was determined by adding 900-950 µl 100 µM S-2238 for thrombin reactions or 100 µM Spectrozyme FXa (American Diagnostica, Greenwich, CT) for factor Xa reactions and monitoring the initial rate of substrate hydrolysis from the linear increase in absorbance at 405 nm. Plots of residual enzyme activity versus molar...
ratio of inhibitor to enzyme were fit by linear regression and the stoichiometry of inhibition (SI) obtained from the abscissa intercept.

Second order rate constants for inhibition of thrombin or factor Xa by N135Q, K114M/N135Q and plasma antithrombins in the absence and presence of natural or 3-O-desulfonated pentasaccharides were measured under pseudo-first order conditions at I 0.025, pH 6.0 or I 0.15, pH 7.4 as in past studies (11,14). Reaction mixtures of 100 µl contained 100-1000 nM of antithrombin and 5-50 nM protease and different concentrations of pentasaccharide. Reaction mixtures were incubated for increasing times at fixed pentasaccharide concentrations or for fixed times at increasing pentasaccharide concentrations at 25°C in polyethylene glycol-coated polystyrene cuvettes and then quenched with 900 µl of 100 µM S-2238 for thrombin or of 100 µM Spectrozyme FXa for factor Xa in I 0.15 sodium phosphate buffer pH 7.4 containing 50-100 µg/ml Polybrene. The residual protease activity was determined from the initial rate of substrate hydrolysis as indicated above. Observed pseudo first-order rate constants (kobs) were obtained from computer fits of the time-dependent or pentasaccharide concentration-dependent decrease in enzymatic activity by an exponential function with a zero activity endpoint (23). Uncatalyzed reactions of protease with antithrombin were shown to be free of traces of heparin by analyses in the absence or presence of 0.1 mg/ml Polybrene. Second order rate constants for uncatalyzed reactions were calculated by dividing kobs by the antithrombin concentration. Pentasaccharide-catalyzed reactions of N135Q and plasma antithrombins with thrombin were determined at saturating pentasaccharide concentrations for the natural pentasaccharide and at subsaturating concentrations for the 3-O-desulfonated pentasaccharide reactions. For the latter, the fitted kobs was corrected for the free antithrombin reaction and then divided by the concentration of antithrombin-pentasaccharide complex as calculated from the quadratic binding equation using the measured K_D for the interaction (19). Second order rate constants for pentasaccharide-catalyzed reactions of antithrombin with factor Xa were determined using two methods. For reactions at I 0.15, pH 7.4, the decrease in protease activity with increasing subsaturating saccharide concentration at a fixed reaction time was fit by an exponential decay function and the second order rate constant was obtained from the equation

\[ k_H = \frac{k_{obs}(t \times [AT]_o)}{([AT]_o + K_D)} \]  

(Eq. 2)

where t is the fixed reaction time, [AT]_o is the antithrombin concentration and K_D is the measured dissociation constant for the antithrombin-pentasaccharide interaction (23). In the second method used for reactions at I 0.025, pH 6, apparent second order rate constants were measured as a function of increasing saccharide concentrations over a range that approached saturation of antithrombin and were then fit by the quadratic binding equation (24). Apparent association rate constants were determined in this case for fixed reaction times from the equation

\[ k_{ass} = \frac{-\ln(v/v_0)}{t \times [AT]_o} \]  

(Eq. 3)

where v and v_0 are the initial rates of substrate hydrolysis of enzyme after reaction for the fixed time, t, or for an unreacted enzyme control, respectively.

RESULTS

Binding of the 3-O-desulfonated-pentasaccharide to antithrombin- To assess the effect of 3-O-desulfonation on the ability of the sequence-specific heparin pentasaccharide, DEFGH (Fig. 1), to allosterically activate antithrombin, we initially compared the binding of natural and 3-O-desulfonated pentasaccharides to antithrombin. Binding was measured by titrating antithrombin tryptophan fluorescence changes that report binding as in past studies. Binding was examined with a β-type N135Q variant of antithrombin missing the N-linked carbohydrate chain at Asn135 that interferes with heparin binding (25-27) and under conditions of low ionic strength (I 0.025), pH 6.0 and 25°C. These conditions were chosen to strengthen heparin binding affinity and thereby allow accurate measurement of the anticipated weak affinity of the 3-O-desulfonated pentasaccharide for antithrombin (15,28). The 3-O-desulfonated pentasaccharide produced a saturable increase in antithrombin tryptophan fluorescence like the natural pentasaccharide under these low
ionic strength and pH conditions (Fig. 2). Nonlinear least squares fitting of variant pentasaccharide binding curves by the equilibrium binding equation yielded an average $K_D$ of $2.8 \pm 0.2 \mu M$. Binding of the natural pentasaccharide under the same conditions was stoichiometric and too tight to measure accurately. An estimated $K_D$ of 40 fM could be obtained by extrapolation of values measured at higher ionic strengths at the same pH (Fig. 3, next section), a value which agrees with the one estimated in previous studies for the N135Q antithrombin-pentasaccharide interaction at I 0.025, pH 6, 25°C (14). These results indicate a dramatic loss in affinity of the pentasaccharide for antithrombin of ~10^8 fold at I 0.025, pH 6 when the 3-O-sulfo group is deleted (Table I). Maximal fluorescence changes induced in antithrombin by the binding of natural and variant pentasaccharides in these experiments were similar (36-37%), indicating that loss of the 3-O-sulfo group did not affect the ability of the bound saccharide to conformationally activate antithrombin (Table I).

**Ionic and Nonionic Contributions to Pentasaccharide Binding**- To determine whether the large loss in pentasaccharide affinity for antithrombin caused by deletion of the 3-O-sulfo group was due to changes in the ionic or nonionic components of the binding interaction, we evaluated the dependence of the observed dissociation constant, $K_{D,obs}$, for the interaction on ionic strength. Fluorescence titrations of the binding of the 3-O-desulfonated pentasaccharide to N135Q antithrombin at pH 6.0 and ionic strengths ranging from 0.025 to ~0.09, or the binding of the natural pentasaccharide at pH 6.0 and higher ionic strengths of 0.3-0.6, showed saturable binding curves with indistinguishable maximal fluorescence enhancements, but with progressive decreases in saccharide affinity for antithrombin with increasing ionic strength. The ionic and nonionic components of the binding were resolved from the linear dependence of log $K_{D,obs}$ on log $[Na^+]$ for the two saccharide interactions (Fig. 2), in accordance with the equation (11,29):

$$\log K_{D,obs} = Z\psi \log [Na^+] + \log K_D' \quad (Eq. 4)$$

where $Z$ is the number of ionic interactions involved in the binding, $\psi$ is the fraction of Na$^+$ ions bound per heparin charge that are released on binding to antithrombin (estimated to be 0.8 (11)), and $K_D'$ is the dissociation constant at 1 M Na$^+$, reflecting the strength of the nonionic interactions. The slopes and intercepts of these plots showed that about six ($5.8 \pm 0.6$) ionic interactions contributed to the binding of the natural pentasaccharide to N135Q antithrombin at pH 6.0 and that nonionic interactions contributed significantly to the binding (log $K_D'$ -5.8±0.2) (Fig. 3, Table II). By contrast, binding of the 3-O-desulfonated pentasaccharide involved 2.0±0.2 ionic interactions and a considerably smaller contribution of nonionic interactions (log $K_D'$ -2.9±0.2). Deletion of the 3-O-sulfo group thus causes a loss of ~4 ionic interactions and an ~800-fold decrease in the strength of nonionic interactions of the pentasaccharide with antithrombin at pH 6.

To extrapolate these findings to physiologic ionic strength and pH we additionally analyzed binding of the 3-O-desulfonated pentasaccharide to N135Q antithrombin at pH 7.4 and ionic strengths in the range 0.025-0.05. Dissociation constants increased from 35±5 $\mu M$ at I 0.025 to 79±18 $\mu M$ at I 0.05 at pH 7.4, values which were ~10-fold weaker than those measured at pH 6 at the same ionic strength. From the linear dependence of log $K_{D,obs}$ on log $[Na^+]$, we estimated that 1.5±0.3 ionic interactions and a log $K_D'$ of -2.4±0.3 for nonionic interactions contributed to the binding at this pH (Fig. 3), providing an estimated $K_{D,obs}$ at physiological ionic strength of 370±220 $\mu M$. Corresponding values for natural pentasaccharide binding to N135Q antithrombin were 5.5±0.5 ionic interactions and log $K_D'$ of -5.1±0.1 for nonionic interactions, yielding an extrapolated $K_{D,obs}$ of 0.0013±0.0006 $\mu M$ at physiological ionic strength. Deletion of the 3-O-sulfo group thus results in a ~$10^5$-fold decreased binding affinity at I 0.15, pH 7.4, 25°C due to the loss of ~4 ionic interactions and a 500-fold affinity loss due to nonionic interactions. Limited studies of the binding of natural and 3-O-desulfonated pentasaccharides to fully glycosylated plasma α-antithrombin at I 0.025, pH 6 indicated an extrapolated $K_D$ of ~1 pM and measured $K_D$ of 17±4 $\mu M$ for natural and
variant pentasaccharide interactions, respectively, indicating comparable losses in pentasaccharide binding energy due to 3-O-desulfonation of ~60% for both α- and β-antithrombin glycoforms.

Rapid kinetics of pentasaccharide binding to antithrombin: The kinetics of binding of the 3-O-desulfonated and natural pentasaccharides to N135Q antithrombin were analyzed at 1~0.04, pH 6.0 under pseudo-first order conditions by monitoring tryptophan fluorescence changes in a stopped-flow instrument as in past studies (11,14). The rapid binding of the natural pentasaccharide to N135Q antithrombin under these conditions limited the analysis to low pentasaccharide concentrations (≤ 2 µM), since rate constants higher than ~500 s⁻¹ could not be reliably measured. A progressive saturation of observed pseudo first order rate constants (k_{obs}) with increasing saccharide concentration was found for the natural pentasaccharide interaction (Fig. 4), that was indicative of a two-step binding process in which an initial binding of the saccharide to antithrombin induces a subsequent conformational change in the inhibitor according to scheme 1 (11,30):

\[
\begin{align*}
\text{AT} + \text{H} & \rightleftharpoons \text{AT} \cdot \text{H} \\
& \rightleftharpoons \text{AT}^* \cdot \text{H}
\end{align*}
\]

Scheme 1

where K₁ is the dissociation constant for an initial rapid equilibrium binding interaction and k₂ and k⁻² are the forward and reverse rate constants for the subsequent conformational change step. The dependence of k_{obs} on saccharide concentration ([H]₀) in figure 4 was fit by the rectangular hyperbolic equation which characterizes this induced conformational change binding mechanism:

\[
k_{\text{obs}} = \frac{k_2 + k_2[H]_0}{([H]_0 + K_1)}
\]  \hspace{1cm} (Eq. 5)

At low heparin concentrations much less than K₁, this equation reduces to:

\[
k_{\text{obs}} = k_2 + k_2[H]_0/K_1 = k_{\text{off}} + k_{\text{on}}[H]_0
\]  \hspace{1cm} (Eq. 6)

Equation 6 indicates that k₂ corresponds to the off-rate constant (k_{off}) and k₂/K₁ represents the on-rate constant (k_{on}) for the overall binding interaction (11). The data were satisfactorily fit by equation 5 which yielded values for K₁ of 0.52±0.05 µM, for k₂ of 530±20 s⁻¹, and from the ratio, k₂/K₁, a k_{on} of 1000±100 µM⁻¹s⁻¹. The fitted value of k₂ was indistinguishable from zero, indicating that k_{off} was too low to determine accurately. Calculation of the expected k⁻² from the relation, k⁻² = K_{D, obs} x k₂/K₁, confirmed an extremely low k_{off} of 4 x 10⁻⁴ s⁻¹.

In contrast to the results for binding of the natural pentasaccharide, k_{obs} for the binding of the 3-O-desulfonated pentasaccharide to N135Q antithrombin showed an initial decrease followed by an increase as the concentration of the pentasaccharide was increased. The initial decline in k_{obs} is diagnostic of an alternative preequilibrium pathway for pentasaccharide activation of antithrombin in which activation occurs by the saccharide selectively binding to a minor fraction of activated antithrombin that already exists in a preequilibrium with native antithrombin (28,31) (Scheme 2). The preference for the preequilibrium activation pathway at low saccharide concentrations implies that the affinity of the variant saccharide for native antithrombin is greatly reduced from that of the natural pentasaccharide. The observation of a shift from a decrease to an increase in k_{obs} at higher saccharide concentrations indicates that the binding mechanism reverts to the induced conformational activation pathway when saccharide concentrations are high enough to favor binding to native antithrombin. The two pathways are components of the general scheme for heparin activation of antithrombin (Scheme 2). In this scheme, K₁, k₂, and k⁻² are the parameters for the induced conformational activation pathway given above, k₃ is the rate constant for preequilibrium conformational activation of antithrombin in the absence of heparin, k⁻₃ is the reverse rate constant for this activation, and K₄ is the dissociation constant for saccharide binding to preequilibrium activated antithrombin (31). Assuming that the binding steps for both pathways are in rapid equilibrium, the dependence of k_{obs} on [H]₀ for this mechanism is given by the equation:

\[
k_{\text{obs}} = (k_3K_1/[H]_0 + k_2)/(1 + K_1/[H]_0 + (k_3 + (k_2[[H]_0/K_4]) / (1 + [H]₀/K₄))
\]  \hspace{1cm} (Eq. 7)
Of the six parameters in this equation, only five are independent, since they are related by the relation:

$$K_1/K_4 = (k_2k_3)/(k_2k_3) = K_3/K_2 \quad \text{(Eq. 8)}$$

where $K_3$ and $K_2$ are the equilibrium constants for the preequilibrium conformational activation and the heparin-induced conformational activation steps of the two binding pathways, respectively. Because of the limited range of saccharide concentrations over which kinetic data was obtainable, fitting of the data by equations 7 and 8 did not allow all parameters to be uniquely determined. However, the observation that $k_{obs}$ showed no evidence of saturation over the range of heparin concentrations examined suggested that the value of $K_1$ for the induced conformational activation pathway was much greater than the highest heparin concentration employed. Under such conditions ($K_1 \gg [H]_o$), equations 7 and 8 reduce to:

$$k_{obs} = (k_3 + (k_2/K_1)[H]_o) \times (1 + k_{-2}k_3/(k_2k_3 + k_{-3}(k_2/K_1)[H]_o)) \quad \text{(Eq. 9)}$$

Assuming our previously determined value for the preequilibrium conformational activation rate constant, $k_3$, of $\sim 10 \text{ s}^{-1}$ under these conditions (28), and fixing $k_{-3}$ at a value of 1000 s$^{-1}$, consistent with the estimated 1% of preequilibrium activated antithrombin (32), a good fit of the data by this equation could be obtained (Fig. 4). Fitted values for $k_3/K_1$ ($k_{on}$) of $44 \pm 2 \mu \text{M}^{-1}\text{s}^{-1}$ and for $k_2$ ($k_{off}$) of $76 \pm 3 \text{ s}^{-1}$ were 25-fold decreased and 200,000-fold increased from corresponding values for natural pentasaccharide binding. The inability to detect saturation of $k_{obs}$ at variant saccharide concentrations of $4 \mu \text{M}$ further implied values for $K_1 > 4 \mu \text{M}$ and for $k_2 > \sim 200 \text{ s}^{-1}$. The 25-fold decrease in $k_{on}$ could therefore be attributed to at least a 10-fold increase in $K_1$ and at most a 2-fold decrease in $k_2$. The fitted values for $k_{on}$ and $k_{off}$ implied an overall dissociation constant ($K_{D,obs}$) of $2 \mu \text{M}$ which was lower than the measured value for $K_{D,obs}$ of $6 \mu \text{M}$ under these conditions (Table 1). However, fixing $K_{D,obs}$ in the range of $2-5 \mu \text{M}$ produced satisfactory fits of the data with only marginal reductions in $k_3/K_1$ to at most $30 \mu \text{M}^{-1}\text{s}^{-1}$, a 2-fold maximal increase in $k_2$ to $150 \text{ s}^{-1}$ and plausible preequilibrium activation ratios ($k_3/k_2$) between 0.1-1%. Kinetic simulations verified that reasonable fits of the data were obtained with the determined parameters even without making the rapid equilibrium assumption. Overall, these findings indicated that loss of the 3-O-sulfo group reduces pentasaccharide affinity for antithrombin through both a decrease in $k_{on}$ and an increase in $k_{off}$, with the latter being overriding.

**Effects of Lys114 mutation on pentasaccharide binding**

Since the X-ray structure of the antithrombin-pentasaccharide complex shows that the 3-O-sulfo group interacts with Lys 114 of antithrombin (13) and mutation of Lys114 produces a substantial binding defect (14), we were interested in comparing the affinity and kinetics of binding of the natural pentasaccharide to a K114M/N135Q antithrombin variant under the same conditions used to analyze the binding of the 3-O-desulfonated pentasaccharide to N135Q antithrombin. $K_{D,obs}$ for the binding of the natural pentasaccharide to the mutant antithrombin was measured to be $190 \pm 3 \text{nM}$ at $I \sim 0.025$ and $252 \pm 80 \text{nM}$ at $I \sim 0.04$, pH 6 and 25°C, in good agreement with values measured previously at $I \sim 0.025$ (14). Values for $Z$ of 2.5 and for log $K_D$' of -3.5 were estimated from this limited data. Analysis of binding at pH 7.4 and ionic strengths between 0.025-0.05 gave $K_{D,obs}$ ranging from $5.3 \pm 0.3 \mu \text{M}$ to $12 \pm 2 \mu \text{M}$, allowing an extrapolated $K_D$ of $60 \pm 20 \mu \text{M}$ to be determined at physiologic ionic strength and pH. Values for $Z$ of 1.5$\pm$0.2 and for log $K_D$ of $-3.2 \pm 0.3$ were obtained for the binding at pH 7.4 from this dependence (Fig. 3). Thus, while the antithrombin-pentasaccharide interaction is considerably weakened by the isosteric replacement of Lys114 with an uncharged Met sidechain, the reduction in affinity is $\sim 10^7$-fold at $I \sim 0.025$, pH 6 and $\sim 50,000$-fold at $I \sim 0.15$, pH 7.4, i.e., $\sim 6$-10-fold less than that caused by removal of the pentasaccharide 3-O-sulfo group. The kinetics of binding of the natural pentasaccharide to K114M antithrombin at pH 6, $I \sim 0.04$ showed a hyperbolic dependence of $k_{obs}$ on saccharide concentration (Fig. 4) indicating that binding exclusively followed the induced conformational change pathway. Values of $K_1$ of $1.7 \pm 1.6 \mu \text{M}$, $k_2$ of $25 \pm 6 \text{ s}^{-1}$ and $k_2$ of $14 \pm 4 \text{ s}^{-1}$ were obtained by fitting the data by equation 5, indicating a $k_{on}$ of...
15±15 μM⁻¹ s⁻¹ and k_off of 14±4 s⁻¹ for the binding interaction. The calculated K_D of 600 nM in this case, obtained from the formula, K_1K_2/(1+K_2), agreed well with the measured value of 520±80 nM. Loss of the positive charge of Lys114 thus has no significant effect on the affinity for native antithrombin in the initial binding step (K_1) but results in major effects on both forward (k_+2) and reverse (k_-2) rate constants in the subsequent conformational activation step, in agreement with previous studies of pentasaccharide binding to a K114A/N135A recombinant antithrombin at a higher ionic strength (14). Notably, the effects of Lys114 mutation on k_-2 were much greater than those due to 3-O-desulfonation, whereas the effects of Lys114 mutation on k_+2 were of the same order of magnitude as those due to 3-O-desulfonation.

Effects of 3-O-desulfonation and Lys114 mutation on pentasaccharide-catalyzed antithrombin-protease reactions- The activating effects of natural and 3-O-desulfonated pentasaccharides on the β-type N135Q and K114M/N135Q antithrombins as well as plasma α-antithrombin were compared by measuring the second order rate constants (k_ass) for reactions of unactivated and pentasaccharide-activated antithrombin with thrombin and factor Xa. Rate constants were measured by discontinuous assays of protease inhibition at I 0.025, pH 6.0, 25°C under pseudo first order conditions as a function of increasing pentasaccharide concentration. The stoichiometries of antithrombin inhibition of thrombin and factor Xa (SI) in the absence and presence of the pentasaccharides were also measured under the same conditions in order to correct rate constants for a competing substrate reaction of antithrombin with proteases (33). This correction, made by multiplying the apparent association rate constant by the SI, is essential since the reaction flux through the substrate pathway increases for pentasaccharide-catalyzed reactions as the ionic strength is decreased (11). Unactivated rate constants were similar to those reported previously under these conditions and were unaffected by the Lys114 mutation (Table 3) (14). Rate constants for natural and variant pentasaccharide-activated reactions of N135Q antithrombin with thrombin and factor Xa measured at subsaturating saccharide concentrations, calculated to reflect the rate constants for protease inhibition by the antithrombin-saccharide complexes using measured K_D's for complex formation, were indistinguishable after correcting for SI (Table 3). By contrast the rate constants for natural pentasaccharide-activated reactions of N135Q and K114M/N135Q antithrombins with the two proteases differed for factor Xa but were similar for thrombin (Table 3). To validate these findings at saturating levels of the saccharides, titrations of the increase in k_ass produced by natural and variant pentasaccharides were performed for N135Q and plasma antithrombin reactions with factor Xa. k_ass increased with increasing saccharide concentration in a saturable manner to reach indistinguishable endpoint values in all cases except for the variant pentasaccharide-activated reaction of plasma antithrombin for which the endpoint was not well determined (Fig. 5 and Table 3). Whereas K_D's could not be determined for natural pentasaccharide activation of N135Q and plasma antithrombins because activation was stoichiometric, a K_D of 2.7±0.1 μM was determined for 3-O-desulfonated pentasaccharide activation of N135Q antithrombin, in excellent agreement with the value measured by equilibrium binding. Assuming the measured K_D of 17±4 μM for the variant pentasaccharide interaction with plasma antithrombin, a fitted endpoint corresponding to full activation was determined. k_ass for the natural pentasaccharide activated reaction of K114M/N135Q antithrombin with factor Xa showed saturable increases to an endpoint k_ass that was ~75% that measured for pentasaccharide activation of N135Q antithrombin with and with a K_D of 190±30 nM that agreed with the value measured by equilibrium binding. Together, these results indicated that deletion of the 3-O-sulfo group does not affect the ability of the bound pentasaccharide to conformationally activate antithrombin whereas mutation of Lys114 modestly reduces the activating effect.

To determine whether these findings could be extrapolated to physiologic pH and ionic strength, we measured the association rate constants and SIs for natural and 3-O-desulfonated pentasaccharide-activated reactions of all forms of antithrombin with factor Xa. Similar to the findings at low pH and ionic strength, the 3-O-desulfonated pentasaccharide produced an
activated rate constant for the N135Q antithrombin reaction with factor Xa that was experimentally indistinguishable from the value produced by the natural pentasaccharide when inhibition rates were measured from 1-10 µM variant saccharide and the antithrombin-saccharide complex concentration was calculated using the extrapolated K_D for the variant pentasaccharide interaction under these conditions. Notably, plasma α-antithrombin resulted in a normal activated rate constant if a K_D for the α-antithrombin-variant saccharide interaction of ~600 µM was assumed. Such a value is comparable to that previously reported under these conditions (15). Also, like the findings at low pH and ionic strength, the natural pentasaccharide produced an activated rate constant for the K114M/N135Q antithrombin reaction with factor Xa that was only modestly reduced from that for the corresponding reaction with N135Q antithrombin.

**DISCUSSION**

Our studies provide new insights into the role of a signature 3-O-sulfo group of the anticoagulant heparin pentasaccharide sequence in the mechanism by which heparin and heparan sulfate glycosaminoglycans allosterically activate antithrombin. By comparing the interactions of natural and 3-O-desulfonated pentasaccharides with both α- and β-type glycoforms of antithrombin over a wide range of ionic strength and pH, we have shown that deletion of the 3-O-sulfo group produces a massive loss in binding affinity that ranges from ~10^7-10^8-fold at pH 6.0, I 0.025 to ~10^4-10^5-fold at pH 7.4, I 0.15 and that corresponds to ~60% of the binding energy. Significantly, our results show that the large affinity loss arises from a pH independent loss of ~4 of 6 ionic interactions as well as a ~500-800-fold decrease in the affinity due to nonionic interactions, corresponding to similar ~30% losses in ionic and nonionic binding energy at physiologic ionic strength. These findings demonstrate that the 3-O-sulfo group is a key mediator of the cooperative network of interactions that contribute to the high-affinity antithrombin-pentasaccharide interaction (9). Such cooperativity has been evident in previous studies from the finding that multiple ionic and nonionic interactions are lost when any one of the three hotspot basic residues of antithrombin in the pentasaccharide binding site are mutated. Remarkably, the antithrombin binding defect resulting from loss of the pentasaccharide 3-O-sulfo group is comparable to or greater than that produced by the loss of other critical sulfo groups in the pentasaccharide including the 6-O-sulfo group of residue D and the N-sulfo group of residue H (15,28,34), as well as that caused by mutating any of the three hotspot residues, Lys114, Lys125 and Arg129, in the pentasaccharide binding site of antithrombin (14,35,36). Not surprisingly, mutation of the binding partner of the 3-O-sulfo group in the X-ray structure of the antithrombin-pentasaccharide complex, Lys114, produces a binding defect that closely approaches, i.e., is ~6-10-fold smaller, than the 3-O-desulfonation.

Despite the greatly reduced affinity of the 3-O-desulfonated pentasaccharide for antithrombin, the variant pentasaccharide was nevertheless able to induce full conformational activation of either α- or β-antithrombin glycoforms as judged from the normal tryptophan fluorescence enhancement and normal enhancement of antithrombin reactivity with factor Xa caused by variant pentasaccharide binding. Previous studies measured the affinity of the 3-O-desulfonated pentasaccharide for the α-glycoform of antithrombin at physiologic pH and ionic strength by equilibrium dialysis and found a K_D of ~500 µM, representing a loss of binding energy comparable to what we have found (15). Surprisingly, later studies reported that the variant saccharide showed a marginal ability either to induce tryptophan fluorescence changes in antithrombin or to enhance rates of antithrombin inhibition of factor Xa (16), results in marked contrast to our findings. While the reason for such discrepant findings is unclear, it should be noted that factor Xa inhibition rates were measured indirectly from fluorescence changes of an active-site bound probe rather than directly as in our studies and both tryptophan and probe fluorescence measurements were made at high protein concentrations expected to cause substantial inner filter fluorescence quenching. We further note that the allosteric activating effects of the heparin pentasaccharide on antithrombin that we have measured in the present study are...
independent of previously reported calcium effects on heparin activation of antithrombin. Such effects reflect the ability of calcium to enhance factor Xa binding to full-length heparins and thereby to promote full-length heparin bridging of antithrombin and factor Xa in a ternary complex (21,24).

Notably, mutation of Lys114 results in somewhat smaller losses in both ionic and nonionic binding energy than 3-O-desulfonation, underscoring a role for the 3-O-sulfo group in the antithrombin-pentasaccharide interaction that is independent of Lys114. This role cannot involve the established ability of the 3-O-sulfo group to induce the adjacent iduronate residue G to favor the high energy skew boat conformation that binds antithrombin with ~1000-fold higher affinity than lower energy chair conformations (37,38). This is because deletion of the 3-O-sulfo group reduces the fraction of skew boat conformer of the iduronate residue only modestly from 64% to 40% (37), a reduction expected to decrease pentasaccharide affinity less than 2-fold under physiologic conditions (38). Moreover, recent studies suggest that 3-O-sulfonated pentasaccharide sequences in longer chain heparins that have the iduronate replaced with a glucuronate retain high affinity for antithrombin (34).

Rapid kinetic studies provided insights into the source of both Lys114 independent and dependent effects of pentasaccharide 3-O-desulfonation. Loss of the 3-O-sulfo group was found to significantly weaken the pentasaccharide interaction with native antithrombin in the initial binding step (K1 increased >10-fold) whereas Lys114 mutation had minimal effects on this step. A role for the pentasaccharide 3-O-sulfo group in recognizing the native antithrombin conformation is supported by previous studies which have shown that the initial binding step is mediated largely by an electrostatically driven interaction of the nonreducing end trisaccharide DEF with native antithrombin (28). Notably, removal of saccharide D from pentasaccharide DEFGH was found to be sufficient to block binding to native antithrombin and favor direct binding to activated antithrombin through the preequilibrium activation pathway. Our present findings similarly show that loss of the 3-O-sulfo group reduces binding to native antithrombin sufficiently to favor activation of antithrombin through the preequilibrium pathway at low saccharide concentrations. Our results therefore suggest that both saccharide D and the 3-O-sulfo group in saccharide F are critically important for the initial electrostatic binding of trisaccharide DEF to native antithrombin.

Loss of the 3-O-sulfo group has a more pronounced effect on the preferential interaction of the pentasaccharide with activated antithrombin in the second conformational activation step, causing a 105-106-fold weakening of this interaction that presumably reflects the loss of interaction with Lys114. The second step defects minimally involve the ability to induce conformational activation (< 2-fold decrease in k+2) and mostly reflect a major loss in the ability to stabilize the activated conformation (k2 increased ~105-fold). However, the activated conformation remains favored over the native conformation (k+2/k-2>> 2), thus accounting for the observed normal enhancements in antithrombin tryptophan fluorescence and reactivity with factor Xa produced by the 3-O-desulfonated pentasaccharide. Mutation of Lys114 similarly greatly weakens the preferential interaction with activated antithrombin in the conformational activation step by ~106-fold. However, this defect involves both a reduced ability to induce conformational activation of antithrombin (k2 decreased ~20-fold) and to stabilize the activated conformation (k2 increased ~40,000-fold). Consequently, the activated conformation is only marginally favored over the native conformation (k+2/k-2 1 to 2), explaining the modestly reduced ability of pentasaccharide to enhance the reactivity of the mutant antithrombin with factor Xa. The 3-O-sulfo group thus contributes similarly to Lys114 to stabilizing antithrombin in the activated state but differs from Lys114 in its contribution to inducing the activated state. Implicit in these findings is that the defects in antithrombin activation produced by pentasaccharide 3-O-desulfonation or Lys114 mutation are fully accounted for by alterations in the equilibrium between native and activated states and not to any changes in the native and activated states themselves.

The X-ray structures of free and pentasaccharide-complexed antithrombin reveal that the unstructured loop preceding the N-terminal end of helix D is induced to form a new P
helix in the activated serpin that positions Lys114 for ionic and hydrogen bonding interactions with the 3-O-sulfo group of the pentasaccharide (13,39). However, Lys114 forms interactions not only with the F saccharide 3-O-sulfo group, but also ionic and hydrogen bonds with the carboxylate and ring oxygen of saccharide G and the N-sulfo group of saccharide H. The importance of these latter interactions is evident from the observation that deletion of the reducing end GH disaccharide causes a loss in pentasaccharide binding energy comparable to that resulting from mutation of Lys114 (28). Formation of the P helix thus represents part of the induced-fit structural changes in antithrombin that allow Lys114 and other basic residues to make complementary interactions with the F, G, and H saccharides in the conformational activation step. Lys114 binding to saccharides F, G and H is a key factor both in driving conformational activation and in stabilizing the activated conformation since mutation of Lys114 greatly affects both forward and reverse conformational activation rate constants (14) Loss of the 3-O-sulfo group likely produces a similar inability to stabilize the activated conformation due to the disruption of Lys114 interactions with saccharides F, G and H, as suggested by the comparable major effects on k\textsubscript{+2} resulting from loss of the 3-O-sulfo group or mutation of Lys114. The interactions lost in this step may include the basic residues, Arg46 and Arg47, that cooperate with Lys114 to bind the GH disaccharide in the conformational activation step (40) as well as Phe 122 which accounts for much of the nonionic binding energy produced in this step (41). The relatively small effect of the 3-O-sulfo group deletion on k\textsubscript{+2} suggests that the 3-O-sulfo group is not important for inducing the activating conformational change. The initial binding of the DEF unit of the pentasaccharide to antithrombin may therefore induce helix P to form and position Lys114 for interactions with saccharides G and H independent of the 3-O-sulfo group but only when the 3-O-sulfo group engages Lys114 can stabilizing interactions with these saccharides be made. The 3-O-sulfo group-Lys114 interaction thus appears pivotal for establishing much of the cooperative network of antithrombin- pentasaccharide interactions that are responsible for the induced-fit interaction of the pentasaccharide with activated antithrombin and the consequent stabilization of the activated conformational state.

Our findings have implications for understanding the greatly reduced antithrombin affinity and activating ability of so-called low-affinity heparins lacking the pentasaccharide sequence (42,43). While this has been thought to reflect the absence of the 3-O-sulfo group marker of this sequence, low affinity heparins show a smaller loss in affinity for antithrombin at physiologic ionic strength and pH (~1000-fold) than that caused by deletion of the 3-O-sulfo group and such heparins only partially induce conformational activation of the serpin. The difference in affinities are understandable because low affinity heparin is a full-length heparin that can bind antithrombin through multiple overlapping sequences extending beyond the pentasaccharide binding site (11,14,42,44,45), resulting in an apparent greater binding affinity. Clues to the reduced activating effect of low affinity heparin come from our previous finding that when the rare E glucuronic acid residue of the minimal DEF activating sequence is replaced by the more common iduronate 2-O-sulfate, only partial conformational activation similar to that of low affinity heparin is observed (28). The preponderance of such trisaccharide sequences in low affinity heparin (46) may thus account for its reduced activating effect, presumably because these sequences preferentially bind and stabilize the native antithrombin conformation over the activated conformation. Given our finding that loss of the glucosamine 3-O-sulfo group does not affect the ability of heparin and presumably also of heparan sulfate to activate antithrombin despite its major effect on affinity, it is noteworthy that mice lacking the enzyme responsible for 3-O-sulfonation of antithrombin binding sequences in heparan sulfate do not show anticoagulant defects (47). Our findings thus suggest the possibility that the abundance of heparan sulfate chains in vivo may allow sufficient activation of antithrombin through sequences lacking the 3-O-sulfo group so as to achieve normal hemostasis.
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FOOTNOTES
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FIGURE LEGENDS

**Fig. 1:** Structures of the natural (1) and 3-O-desulfonated (2) heparin pentasaccharides. Pentasaccharide structures are shown from nonreducing to reducing ends with the saccharides designated DEFGH for historical reasons.
Fig. 2: Equilibrium binding of natural and 3-O-desulfonated pentasaccharides to N135Q antithrombin at pH 6, 25°C and different ionic strengths. Shown are titration curves for the binding of natural (left panel) and 3-O-desulfonated (right panel) pentasaccharides to 1 μM antithrombin at ionic strengths of 0.35 (○), 0.45 (●), 0.55 (▲), and 0.65 (△) for the natural pentasaccharide and of 0.025 (●), 0.037 (○), 0.062 (▲), and 0.087 (△) for the 3-O-desulfonated pentasaccharide. Binding was monitored from relative changes in antithrombin tryptophan fluorescence and binding curves were fit by the quadratic binding equation (solid lines) to obtain measured KDs and maximal relative fluorescence changes (Table 1) as described in Experimental Procedures.

Fig. 3: Sodium ion concentration dependence of observed dissociation equilibrium constants (K_D,obs) for the binding of natural and 3-O-desulfonated pentasaccharides (H5) to N135Q and K114M/N135Q antithrombins at pH 6 and pH 7.4, 25 ºC. ○, natural H5, N135Q AT, pH 6.0; ▲, natural H5, N135Q AT, pH 7.4; ◇, 3-O-desulfonated H5, N135Q AT, pH 6.0; △, 3-O-desulfonated H5, N135Q AT, pH 7.4; □, natural H5, K114M/N135Q AT, pH 7.4. K_D,obs values are averages ± S.E. of two or three determinations. Solid lines represent linear regression fits of the data.

Fig. 4: Kinetics of binding of natural and 3-O-desulfonated pentasaccharides to N135Q and K114M/N135Q antithrombins. Shown is the pentasaccharide concentration dependence of observed pseudo-first-order rate constants (k_obs) for the binding of the natural pentasaccharide to N135Q and K114M/N135Q antithrombins (left panel) and for the binding of the 3-O-desulfonated (Δ3OSO3) pentasaccharide to N135Q antithrombin (right panel) at 25 ºC, pH 6.0 and I 0.04. ○, natural H5, N135Q AT; ▲, desulfonated H5, N135Q AT; ◇, natural H5, K114M/N135Q AT. Each rate constant reflects an average of ~ 20 individual measurements. Solid lines are fits to equations given in the text.

Fig. 5: Kinetic titrations of the enhancement of antithrombin reactivity with factor Xa produced by natural and 3-O-desulfonated pentasaccharides at I 0.025, pH 6. Shown are titrations of the increase in the apparent k_{ass} for reactions of N135Q (○, ▲, left and middle panels), K114M/N135Q (■, right panel) and plasma (○, △, left and middle panels) antithrombins with factor Xa produced by the natural pentasaccharide (left and right panels) or by the 3-O-desulfonated (Δ3OSO3) pentasaccharide (middle panel) as a function of the pentasaccharide concentration. Apparent k_{ass} values were calculated from measured values of kObs for reactions of ~100 nM antithrombin with 5 nM factor Xa in the presence of the indicated concentrations of saccharide as described in Experimental Procedures. Solid lines are fits of data by the quadratic binding equation in which K_D was fixed at measured values for natural pentasaccharide interactions with N135Q and plasma antithrombins and for the 3-O-desulfonated pentasaccharide interaction with plasma antithrombin. K_D was fit as a parameter for the 3-O-desulfonated pentasaccharide activated reaction of N135Q antithrombin or for the natural pentasaccharide activated reaction of K114M/N135Q antithrombin. The stoichiometry of pentasaccharide interaction with antithrombins was fit in reactions of the left panel and these values were assumed in fits in the middle panel. A stoichiometry of 1 was assumed for the fit in the right panel. The maximum k_{ass} was a fitted parameter in all cases.
**Table 1:** Dissociation equilibrium constants and maximum tryptophan fluorescence changes for the binding of 3-O-desulfonated and natural pentasaccharides to N135Q antithrombin at pH 6.0, 25 ºC and varying ionic strengths. Equilibrium binding titrations of antithrombin with natural and variant pentasaccharides were fit by the quadratic binding equation given in the text to obtain values for \( K_{D,\text{obs}} \) and \( \Delta F_{\text{max}}/F_0 \). The table provides average values ± S.E. from two or three fluorescence titrations.

| Pentasaccharide | Ionic strength | \( K_{D,\text{obs}} \) (M) | \( \Delta F_{\text{max}}/F_0 \) |
|-----------------|----------------|--------------------------|------------------|
| natural         | 0.025          | \( 4 \times 10^{-14} \) a | 0.37 ± 0.03      |
|                 | 0.35           | \( 11 \pm 2 \times 10^{-9} \) | 0.32 ± 0.01      |
|                 | 0.45           | \( 32 \pm 10 \times 10^{-9} \) | 0.36 ± 0.03      |
|                 | 0.55           | \( 114 \pm 24 \times 10^{-9} \) | 0.31 ± 0.01      |
|                 | 0.65           | \( 174 \pm 17 \times 10^{-9} \) | 0.35 ± 0.02      |
| desulfonated    | 0.025          | \( 2.8 \pm 0.2 \times 10^{-6} \) | 0.36 ± 0.02      |
|                 | 0.037          | \( 6.2 \pm 0.7 \times 10^{-6} \) | 0.40 ± 0.02      |
|                 | 0.062          | \( 11 \pm 2 \times 10^{-6} \) | 0.35 ± 0.04      |
|                 | 0.087          | \( 27 \pm 3 \times 10^{-6} \) | 0.38 ± 0.01      |

a Value obtained by extrapolation of values obtained at higher ionic strengths using equation 4 and the fitted parameters in Table 2.
Table 2: Ionic and nonionic contributions to the interactions of natural and 3-0-desulfonated pentasaccharides with N135Q and K114M/N135Q antithrombins at pH 6 and pH 7.4, 25°C. The number of ionic interactions (Z) and the nonionic contribution (log $K_D'$) to the binding of pentasaccharides to antithrombins were determined from computer-fitted values of the slope and intercept, respectively, of linear plots of log $K_{D,obs}$ versus log [Na+] shown in figure 3 as described in the text.

| Antithrombin   | Pentasaccharide | pH  | Z     | log $K_D'$     |
|----------------|----------------|-----|-------|----------------|
| N135Q          | natural        | 6.0 | 5.8±0.6 | -5.8±0.2       |
|                | desulfonated   |     | 2.0±0.2 | -2.9±0.2       |
|                | natural        | 7.4 | 5.5±0.2 | -5.1±0.1       |
|                | desulfonated   |     | 1.5±0.3 | -2.4±0.3       |
| K114M/N135Q    | natural        | 6.0 | 2.5   | -3.5           |
|                |                | 7.4 | 1.5±0.2 | -3.2±0.3       |
## Table 3: Association rate constants (k\textsubscript{ass}) and stoichiometries of inhibition (SI) for reactions of N135Q, K114M/N135Q and plasma antithrombins with thrombin and factor Xa in the absence and presence of normal (H5) or 3-O-desulfonated pentasaccharides (Δ3OSO\textsubscript{3}H5)

Apparent association rate constants (k\textsubscript{ass}) and stoichiometries of inhibition (SI) for antithrombin-protease reactions were measured as described in Experimental Procedures. The product of the apparent association rate constant and SI (k\textsubscript{ass} x SI) corrects for the fraction of antithrombin reacting through the substrate pathway and thus represents the rate constant for reaction through the inhibitory pathway.

| Protease | Antithrombin | Pentasaccharide | k\textsubscript{ass} (M\textsuperscript{-1}s\textsuperscript{-1}) | SI (mol l/mol E) | k\textsubscript{ass} x SI (M\textsuperscript{-1}s\textsuperscript{-1}) |
|----------|--------------|----------------|----------------|----------------|----------------------------------|
| Thrombin  |              |                |                |                |                                  |
| N135Q    | None         |                | 4.7±0.1 x 10\textsuperscript{2} | 1.4±0.1 | 6.6±0.6 x 10\textsuperscript{2} |
| N135Q    | H5           |                | 1.2±0.1 x 10\textsuperscript{3} | 1.6±0.2 | 1.9±0.4 x 10\textsuperscript{3} |
| N135Q    | Δ3OSO\textsubscript{3}-H5 | 1.7±0.4 x 10\textsuperscript{3} | 1.2±0.1 | 2.0±0.6 x 10\textsuperscript{3} |
| K114M/N135Q | None     |                | 7.2±1.0 x 10\textsuperscript{2} | 0.94±0.08 | 6.8±1.5 x 10\textsuperscript{2} |
| K114M/N135Q | H5             |                | 1.5±0.2 x 10\textsuperscript{3} | 1.1±0.1 | 1.7±0.4 x 10\textsuperscript{3} |
| Factor Xa |              |                |                |                |                                  |
| N135Q    | None         |                | 5.9±0.6 x 10\textsuperscript{2} | 1.3±0.1 | 7.7±1.4 x 10\textsuperscript{2} |
| N135Q    | H5           |                | 6.6±0.2 x 10\textsuperscript{4} | 1.3±0.1 | 8.6±0.9 x 10\textsuperscript{4} |
| N135Q    | Δ3OSO\textsubscript{3}-H5 | 6.4±0.1 x 10\textsuperscript{4} | 1.3±0.1 | 8.3±0.8 x 10\textsuperscript{4} |
| K114M/N135Q | None     |                | 5.8±0.8 x 10\textsuperscript{2} | 1.0±0.2 | 5.8±2.0 x 10\textsuperscript{2} |
| K114M/N135Q | H5             |                | 5.1±0.2 x 10\textsuperscript{4} | 1.2±0.1 | 6.1±0.7 x 10\textsuperscript{4} |
| Plasma   | None         |                | 1.5±0.1 x 10\textsuperscript{2} | 1.3±0.1 | 2.0±0.3 x 10\textsuperscript{2} |
| Plasma   | H5           |                | 7.0±0.5 x 10\textsuperscript{4} | 1.2±0.1 | 8.4±1.3 x 10\textsuperscript{4} |
| Plasma   | Δ3OSO\textsubscript{3}-H5 | 6.5±0.2 x 10\textsuperscript{4} | 1.3±0.1 | 8.4±0.9 x 10\textsuperscript{4} |
| Conditions: | I 0.025, pH 6.0, 25ºC |                  |                |                |                                  |
| Factor Xa |              |                |                |                |                                  |
| N135Q    | None         |                | 5.9±1.7 x 10\textsuperscript{3} | 1.2±0.1 | 7.1±2.6 x 10\textsuperscript{3} |
| N135Q    | H5           |                | 5.6±0.2 x 10\textsuperscript{5} | 1.5±0.1 | 8.4±0.9 x 10\textsuperscript{5} |
| N135Q    | Δ3OSO\textsubscript{3}-H5 | 9.9±6.6 x 10\textsuperscript{5} | 1.4±0.1 | 1.4±1.0 x 10\textsuperscript{6} |
| K114M/N135Q | None     |                | 3.9±2.8 x 10\textsuperscript{3} | 0.91±0.02 | 3.5±2.6 x 10\textsuperscript{3} |
| K114M/N135Q | H5             |                | 3.4±1.5 x 10\textsuperscript{5} | 1.2±0.1 | 4.1±2.2 x 10\textsuperscript{5} |

\( a \) Values for antithrombin-pentasaccharide complex reactions with factor Xa were obtained from fitted endpoints in Fig. 5.

\( b \) Value taken from reference 35

\( c \) The measured KD of 17±4 µM for the variant pentasaccharide interaction was assumed in the fitting of k\textsubscript{ass}.

\( d \) Values determined from k\textsubscript{obs} for reactions with 1-10 µM pentasaccharide and calculated antithrombin-pentasaccharide complex concentrations assuming extrapolated KDs for complex formation given in the text. The errors in extrapolated KDs account for the large uncertainties in k\textsubscript{ass}.
Fig. 3

![Graph showing the relationship between log $K_{D,obs}$ (M) and log [Na$^+$] (M).](http://www.jbc.org/Downloaded from)
Fig. 4

![Graph showing the relationship between $k_{obs}$ (s$^{-1}$) and [Natural pentasaccharide] (µM).]
Fig. 5

Plasma or N135Q AT

K114M/N135Q AT
Scheme 2

Induced conformational activation pathway

AT + H5 \( \xrightarrow{k_{-3}} \) AT \( \bullet \) H5
\( \xrightarrow{k_3} \)

AT* + H5 \( \xrightarrow{k_{-2}} \) AT* \( \bullet \) H5
\( \xrightarrow{k_2} \)

Preequilibrium activation pathway

\( K_1 \)

\( K_4 \)
The signature 3-O-sulfo group of the anticoagulant heparin sequence is critical for heparin binding to antithrombin but is not required for allosteric activation

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