Importance of Lysine 125 for Heparin Binding and Activation of Antithrombin†

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ABSTRACT: The anticoagulant sulfated polysaccharide, heparin, binds to the plasma coagulation proteinase inhibitor, antithrombin, and activates it by a conformational change that results in a greatly increased rate of inhibition of target proteinases. Lys125 of antithrombin has previously been implicated in this binding by chemical modification and site-directed mutagenesis and by the crystal structure of a complex between antithrombin and a pentasaccharide constituting the antithrombin-binding region of heparin. Replacement of Lys125 with Met or Gln in this work reduced the affinity of antithrombin for full-length heparin or the pentasaccharide by 150−600-fold at $I = 0.15$, corresponding to a loss of 25−33% of the total binding energy. The affinity decrease was due both to disruption of approximately three ionic interactions, indicating that Lys125 and two other basic residues of antithrombin act cooperatively in binding to heparin, and to weakened nonionic interactions. The mutations caused a 10−17-fold decrease in the affinity of the initial, weak binding step of the two-step mechanism of heparin binding to antithrombin. They also increased the reverse rate constant of the second, conformational change step by 10−50-fold. Lys125 is thus a major heparin-binding residue of antithrombin, contributing an amount of binding energy comparable to that of Arg129, but less energy than Lys114. It is the first residue identified so far that has a critical role in the initial recognition of heparin by antithrombin, but also appreciably stabilizes the heparin-induced activated state of the inhibitor. These effects are exerted by interactions of Lys125 with the nonreducing end of the heparin pentasaccharide.

The plasma proteinase inhibitor, antithrombin, a major regulator of blood clotting, is essential for life. Decreased amounts of antithrombin in humans are associated with an increased risk for thrombosis (*1*), and deletion of the gene in mice, resulting in the complete absence of the protein, leads to embryonic lethality (*2*). Antithrombin exerts its effect by inhibiting most coagulation proteinases, although the inhibition of thrombin and factor Xa is of greatest physiological relevance (*1*, *3*, *4*). It is an inhibitor of the serpin family and inactivates target proteinases by a mechanism typical of such inhibitors. The proteinase initially attacks a reactive bond in an exposed loop of the serpin as a regular substrate. However, normal hydrolysis of the acyl intermediate formed during this reaction is interrupted by the rapid insertion of the N-terminal part of the opened loop into a major β-sheet of the inhibitor, the A sheet. The proteinase is tethered to the end of the inserting loop segment by an acyl bond and is therefore translocated to the opposite pole of the serpin. In this position, it is compressed against the main body of the serpin, causing it to be inactivated by an extensive distortion of its structure (*3*−*7*).

The moderate rates of antithrombin inactivation of thrombin and factor Xa are greatly increased to diffusion-limited rates by the sulfated glycosaminoglycan, heparin (*3*, *4*). Physiologic inhibition of these enzymes by antithrombin may therefore require heparin, or a heparin-like polysaccharide, as a cofactor (*4*, *8*). The ability of heparin to increase the inhibition rate of both enzymes is dependent on the presence of a specific pentasaccharide sequence in the polysaccharide chain (*9*, *10*). Heparin chains containing this pentasaccharide bind tightly to antithrombin by a two-step mechanism, in which an initial, low-affinity recognition complex is formed in the first step and a conformational change that increases the binding affinity and activates the inhibitor is induced in the second step (*11*, *12*). The X-ray structures of antithrombin

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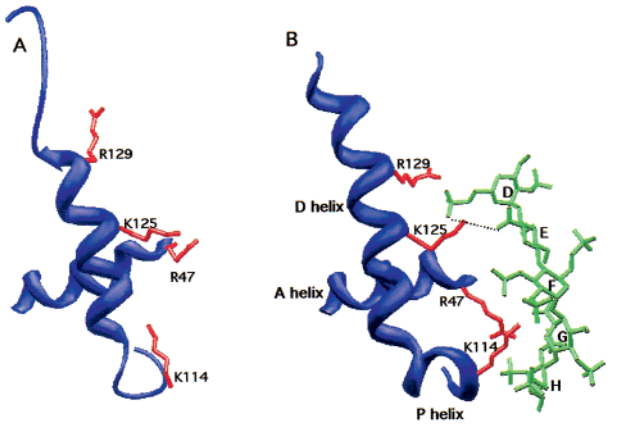

FIGURE 1: Closeup of the heparin binding site of antithrombin. (A) Antithrombin alone and (B) the antithrombin−pentasaccharide complex. The N-terminal end of the A helix, the D helix, and the P helix in the antithrombin−pentasaccharide complex and the corresponding regions in antithrombin alone are shown in blue. The side chains of the major pentasaccharide-binding residues (Arg47, Lys114, Lys125, and Arg129) are drawn in red. The pentasaccharide, DEFGH, with individual saccharide units denoted in alphabetical order from the nonreducing end, is represented in green. The interactions between Lys125 and the D and E units of the pentasaccharide are denoted with dotted lines. Drawn from PDB structures 2ant and 1azx (*13*, *14*).

and its complex with a synthetic heparin pentasaccharide (*13*, *14*) indicate that this change involves a substantial rearrangement of the structure of the inhibitor. The D helix of the protein is tilted and elongated by 1.5 turns; a new, short α-helix, the P helix, is formed, and the A sheet is contracted. These changes lead to the expulsion of two residues at the base of the reactive-bond loop from their partially buried location in the A sheet, making the reactive bond more accessible. This conformational change is necessary and sufficient for accelerated inhibition of factor Xa, whereas thrombin inhibition requires bridging of antithrombin and the proteinase by both reactants binding to the same, ≥18-saccharide long, pentasaccharide-containing heparin chain (*3*, *4*).

Previous studies of chemically modified antithrombins and of natural and recombinant antithrombin mutants have identified several residues of the inhibitor, primarily Arg47, Lys114, Lys125, and Arg129, as being important for heparin binding (*15*−*21*). The X-ray structure of the antithrombin−pentasaccharide complex shows that these residues are located together on the A, D, and P helices of antithrombin and are the main residues forming the heparin pentasaccharide binding site (Figure 1) (*13*, *14*). The roles of three of these residues (Arg47, Lys114, and Arg129) in heparin binding have recently been investigated in detail by site-directed mutagenesis (*22*−*24*). Substitution of Lys114 led to an ∼10^5-fold loss in heparin affinity, reflecting a major contribution of this residue to the binding. In contrast, mutation of Arg129 and Arg47 decreased the affinity only 400−2500- and 20−30-fold, respectively, showing that these residues are appreciably less important. Lys114 and Arg129, but not Arg47, were each shown to act cooperatively with at least one other basic residue in binding of the heparin pentasaccharide. All three residues participate minimally in the first step of heparin binding but instead contribute predominantly to the second step, mainly by decreasing the reverse rate constant of this step. However, Lys114 also appreciably increases the forward rate constant of the second step. Analogous substitutions of the fourth putative major heparin-binding residue, Lys125, have been reported to lead to discrepant 30−150-fold (*18*) and ∼2-fold (*19*) reductions in heparin affinity. Moreover, detailed studies of the functional role of this residue in the binding are lacking.

In this work, we have characterized the role of Lys125 of antithrombin in heparin binding and activation of the inhibitor by mutation of this residue to Met or Gln. The results confirm that Lys125 is a major heparin-binding residue, contributing an amount of binding energy comparable to that of Arg129. Together with the latter residue and Lys114, it forms the core of a cooperative network of residues contributing both ionic and nonionic interactions with the heparin pentasaccharide. Notably, Lys125 is the first of all residues investigated so far to have a critical role in the initial recognition of heparin, increasing the affinity of the first binding step by 10−17-fold. In addition, Lys125 is important in antithrombin activation by decreasing the reverse rate constant of the second binding step by 10−50-fold, thereby appreciably stabilizing the conformationally altered state of the inhibitor.

MATERIALS AND METHODS

Antithrombin Variants. Antithrombin variants with substitutions of Lys125 with Met or Gln were produced by site-directed mutagenesis with the previously characterized Asn135 to Ala antithrombin variant as the base molecule and were expressed in a baculovirus system (*21*−*23*, *25*, *26*). The N135A[1] control variant, expressed in the same system, and the K125M/N135A and K125Q/N135A variants were purified by affinity chromatography on a heparin−agarose column essentially as described in detail earlier (*21*−*24*, *26*). The latter two variants required further purification by anion-exchange chromatography on a MonoQ HR 5/5 column (Amersham Pharmacia Biotech) (*24*).

A Lys125 to Met antithrombin variant was also produced by site-directed mutagenesis on a wild-type background and was expressed in a baby hamster kidney cell system (*18*, *27*−*29*). Similarly expressed recombinant wild-type antithrombin (*27*−*29*) and an Asn135 to Gln antithrombin variant (*24*, *30*) were used as controls for this variant. Purification of the recombinant wild-type inhibitor and the K125M variant by heparin affinity chromatography gave three peaks, of which peak III (*29*), eluting at the highest salt concentration, was used in this work. Analogous chromatography of the N135Q variant gave two peaks, the last-eluting one being used (*24*). Each of the three antithrombin forms was further purified by successive chromatographies on DEAE-Sepharose and Sephacryl S-200 (Amersham Pharmacia Biotech), as described previously (*31*).

The purity of the antithrombin preparations was analyzed by SDS−PAGE with the Tricine or Laemmli buffer systems

[1] Abbreviations: H26, full-length heparin with high affinity for antithrombin and containing ∼26 saccharide units; H5, antithrombin-binding heparin pentasaccharide; K_d, dissociation equilibrium constant; k_{obs}, observed pseudo-first-order rate constant; k_{on}, overall association rate constant; k_{off}, overall dissociation rate constant; K125M and K125Q, mutants with substitution of Lys125 with Met and Gln, respectively; N135A and N135Q, mutants with substitution of Asn135 with Ala and Gln, respectively; PAGE, polyacrylamide gel electrophoresis; rWT-III, recombinant wild-type, form III; SDS, sodium dodecyl sulfate.

(*32*, *33*) and by nondenaturing PAGE with the Laemmli buffer system. Concentrations of all antithrombin forms were determined from the absorbance at 280 nm with the use of the molar absorption coefficient of plasma antithrombin [37 700 M^{-1} cm^{-1} (*34*)]. In some cases, concentrations of dilute (less than ~2 μM) samples of the K125M/N135A and K125Q/N135A variants were instead determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) with the N135A variant as the standard.

Proteinases and Saccharides. Human α-thrombin was a gift from J. Fenton (New York State Department of Health, Albany, NY), and human factor Xa (predominantly the α form) was purified as described elsewhere (*35*). The concentrations of the enzymes were based on active-site titrations, which showed that thrombin and factor Xa were >90 and ~70% active, respectively. The synthetic antithrombin-binding heparin pentasaccharide (*10*) and tetra- or trisaccharides lacking the reducing or nonreducing ends of this pentasaccharide (DEFG*, DEF, and EFGH′ in ref *36*) were generous gifts from M. Petitou (Sanofi Recherche, Toulouse, France). Full-length heparin with a high affinity for antithrombin and with a reduced polydispersity and an average molecular weight of ~8000, i.e., containing ~26 saccharide units, was isolated from commercial heparin (*12*, *26*, *37*). Concentrations of all saccharides except the EFGH′ tetrasaccharide were obtained from stoichiometric titrations, monitored by tryptophan fluorescence, of plasma antithrombin with the saccharides at pH 7.4 or 6.0 (*31*, *36*). The concentration of the EFGH′ tetrasaccharide was determined by weight.

Experimental Conditions. All experiments were carried out at 25.0 ± 0.2 °C in 20 mM sodium phosphate, 100 μM EDTA, and 0.1% (w/v) poly(ethylene glycol) 8000, adjusted to pH 6.0 or 7.4. The ionic strength of this buffer is 0.025 and 0.05 at the two pH values, respectively, and NaCl was added if higher ionic strengths were desired. Most analyses were carried out at pH 7.4 and $I = 0.15$.

Stoichiometries and Affinities of Heparin Binding. Stoichiometries and dissociation equilibrium constants, K_d, for the binding of oligosaccharides or full-length heparin to the antithrombin variants were measured by titrations monitored by the enhancement of tryptophan fluorescence induced by the interaction, as described previously (*22*, *23*, *26*, *31*). Binding stoichiometries were measured at pH 7.4 and $I = 0.05-0.15$ for most variants and also at pH 6.0 and $I = 0.025$ for the K125M/rWT-III variant, in all cases at antithrombin concentrations, determined by absorbance, >10-fold above K_d. Binding affinities were determined at pH 6.0 or 7.4 and different ionic strengths at concentrations of active antithrombin ~5-fold below to ~3-fold above K_d. All titrations were carried out in an SLM 4800S or 8000C spectrofluorometer (SLM Instruments, Rochester, NY) with excitation and emission wavelengths of 280 and 340 nm, respectively. The data were fitted to the equilibrium binding equation by nonlinear least-squares analysis (*31*).

Kinetics of Heparin Binding. The kinetics of binding of pentasaccharide or full-length heparin to the antithrombin variants were analyzed under pseudo-first-order conditions at pH 7.4 and different ionic strengths by monitoring the increase in protein fluorescence in an SX-17MV stopped-flow instrument (Applied Biophysics, Leatherhead, U.K.) as in earlier work (*12*, *22*, *23*, *26*). Saccharide concentrations were at least 5-fold and in most cases 10-fold higher than concentrations of active antithrombin. The fluorescence was measured with an excitation wavelength of 280 nm and an emission cutoff filter with ~50% transmission at 320 nm. Progress curves were fitted to a single-exponential function to give the observed pseudo-first-order rate constant, k_{obs}. Typically, 8−16 fluorescence traces were acquired for each rate constant determination and k_{obs} values from these traces averaged.

Stoichiometries and Kinetics of Proteinase Inactivation. Stoichiometries of inhibition of active-site-titrated human α-thrombin by the antithrombin variants were measured essentially as described in detail previously (*22*, *23*, *31*). Increasing amounts of antithrombin variant were added to a series of samples of thrombin at a constant concentration of 0.05 or 0.5 μM in pH 7.4, $I = 0.15$ buffer. The residual activity of the enzyme was then determined after 10−24 h (for 0.05 μM thrombin) or 1−2 h (for 0.5 μM thrombin) from the initial rate of hydrolysis of the substrate, S-2238 (D-Phe-Pip-Arg-*p*-nitroanilide; Chromogenix, Mölndal, Sweden), monitored by absorbance at 405 nm. The inhibition stoichiometries were obtained from the intercept on the abscissa of plots of the residual enzyme activity versus the molar ratio of inhibitor to enzyme, based on antithrombin concentrations determined by absorbance (*31*).

Second-order rate constants for inhibition of human α-thrombin or factor Xa by the antithrombin variants in the absence and presence of pentasaccharide or full-length heparin were measured under pseudo-first-order conditions in pH 7.4, $I = 0.15$ buffer, as in earlier work (*22*, *23*, *26*, *31*, *36*, *38*). Reaction mixtures contained 25−200 nM active antithrombin, 5−10 nM proteinase, and in most cases 0−10 nM pentasaccharide or full-length heparin. However, in the analyses of the pentasaccharide-catalyzed factor Xa inactivation by the K125M/rWT-III variant, the pentasaccharide concentration was 100−200 nM. After different reaction times, aliquots were diluted into 100 μM S-2238 for thrombin or 100−200 μM Spectrozyme FXa (American Diagnostica, Greenwich, CT) for factor Xa, and the residual proteinase activity was determined. Observed pseudo-first-order rate constants, k_{obs}, were obtained by fitting the dependence of this activity with time to a single-exponential function with an end point of zero activity (*31*). Second-order rate constants for uncatalyzed reactions were obtained by dividing k_{obs} with the antithrombin concentration. Most such rate constants for pentasaccharide- and full-length heparin-catalyzed reactions were derived from the least-squares slopes of the linear dependence of k_{obs} on the concentration of the antithrombin−saccharide complex, calculated from measured K_d values (*26*, *36*, *38*). Alternatively, the rate constant for the pentasaccharide-catalyzed factor Xa inactivation by the K125M/rWT-III variant was calculated from k_{obs} measured at single pentasaccharide concentrations by first subtracting k_{obs} for the uncatalyzed reaction and then dividing by the calculated concentration of the antithrombin−pentasaccharide complex (*26*, *36*, *38*), with several such values averaged. As the catalyzed rate constants in both procedures are based on the concentrations of the antithrombin−saccharide complexes, they represent the rate constants at saturation of antithrombin with the saccharides.

RESULTS

Purification and Homogeneity of Antithrombin Variants. K125M and K125Q antithrombin variants were expressed on an N135A background in a baculovirus system, as in our previous studies of the roles of Arg47, Lys114, and Arg129 of the inhibitor in heparin binding (*22*−*24*). The N135A substitution leads to an antithrombin form which has an increased heparin affinity due to the absence of an oligosaccharide side chain on Asn135 and thus corresponds to the β isoform of plasma antithrombin (*25*, *26*). More importantly, this substitution eliminates the heterogeneity in heparin binding affinity resulting from partial glycosylation of Asn135 (*25*, *39*). Both the K125M and K125Q substitutions lead to loss of the positive charge of Lys125 but introduce side chains approximately isosteric with that of Lys. The N135A control variant eluted at ∼2.5 M NaCl in heparin affinity chromatography and was sufficiently homogeneous to be used without further purification (*25*, *26*). In contrast, the K125M/N135A and K125Q/N135A variants eluted at 0.6−0.7 M NaCl together with other proteins and therefore required further purification by anion-exchange chromatography.

A K125M antithrombin variant was also expressed on a wild-type background in a baby hamster kidney cell system, which differs from the baculovirus system in introducing longer, sialic acid-containing carbohydrate side chains (*25*, *29*, *40*). Heparin affinity chromatography of both wild-type antithrombin and variants of the inhibitor on the wild-type background expressed in this system gives three peaks (*29*), of which form III, eluting at the highest salt concentration, was used in this work. This form lacks carbohydrate on Asn135, is nonfucosylated, and, like the antithrombin forms expressed on an N135A background, corresponds to β-antithrombin in plasma (*29*, *40*, *41*). The rWT-III antithrombin control and the K125M/rWT-III variant eluted at ∼1.8 and ∼0.7 M NaCl, respectively. In addition, an N135Q control variant intentionally engineered to lack carbohydrate on Asn135 was expressed in the same system. Purification of this variant by affinity chromatography on immobilized heparin gave two peaks, the nonfucosylated form, having the highest heparin affinity and eluting at ∼1.9 M NaCl, being used (*24*). Both rWT-III antithrombin and the N135Q variant, which are structurally equivalent, served as controls in most experiments, which showed that they also are functionally similar (see below). Therefore, only one of the two was used as a control in some cases. All three antithrombin forms were further purified by anion-exchange and gel chromatography.

All final antithrombin preparations were more than 95% homogeneous on SDS−PAGE and nondenaturing PAGE. The K125 mutants migrated in a manner indistinguishable from those of their controls on SDS−PAGE but had slightly higher mobilities than the controls under nondenaturing conditions at alkaline pH, in agreement with the loss of positive charge. Moreover, rWT-III antithrombin and the K125M/rWT-III variant migrated faster than the fully glycosylated form II of recombinant wild-type antithrombin (*29*) but indistinguishably from the N135Q variant on SDS−PAGE, consistent with the absence of a carbohydrate side chain. The stoichiometries of heparin binding to the variants were determined by titrations, monitored by the increase in tryptophan fluorescence induced by the binding, with full-length heparin in the case of the baculovirus-expressed variants and with pentasaccharide for the variants expressed in the mammalian system. These stoichiometries were 0.7−0.8, 0.5−1.0, 0.5−0.8, 0.8−1.0, 0.8−1.0, and 0.7−0.8 for the different preparations of the N135A, K125M/N135A, K125Q/N135A, rWT-III, N135Q, and K125M/rWT-III variants, respectively, that were used. All antithrombin forms exhibited fluorescence increases of ∼30% on saturation with the saccharides in the stoichiometric titrations. The corresponding thrombin to antithrombin binding stoichiometries, measured by titrations monitored by the loss of thrombin activity, were 0.6−0.7, 0.6−0.7, 0.7−0.8, 0.8−0.9, 0.8−0.9, and 0.7−0.8, respectively. As in previous studies with other recombinant antithrombin variants (*22*−*24*, *26*), all preparations thus contained some inactive, most likely latent (*42*), inhibitor, the N135A or -Q and rWT-III controls being most active. In subsequent studies of heparin binding and proteinase inhibition, concentrations of active antithrombin in each preparation of the N135A, K125M/N135A, and K125Q/N135A variants were those derived from the heparin and thrombin binding stoichiometries, respectively. However, in both types of analyses, concentrations of active rWT-III antithrombin and the N135Q and K125M/rWT-III variants were obtained from the thrombin binding stoichiometries.

Affinity of Pentasaccharide and Full-Length Heparin Binding. Dissociation equilibrium constants, K_d, for the binding of pentasaccharide or full-length heparin to the three K125 antithrombin mutants were measured by titrations, monitored by tryptophan fluorescence, at pH 7.4, $I = 0.15$, and antithrombin concentrations approximating K_d (Table 1). Due to the tight binding of the two saccharides to the three control variants under these conditions, however, the affinities of these interactions had to be obtained by extrapolation of values measured at higher ionic strengths (Table 1; see further below). The rWT-III and N135Q control variants bound pentasaccharide with comparable affinities, reflecting equivalent functional properties. As in previous work (*12*, *22*−*24*, *26*), all antithrombin forms bound full-length heparin 3−10-fold more tightly than the pentasaccharide. Mutation of Lys125 caused an appreciable decrease in the affinity of antithrombin for both pentasaccharide and full-length heparin, the decrease varying between 150- and 600-fold for the three mutants, largely due to the somewhat uncertain control values. However, small differences in affinity between the mutants were apparent. The K125M/N135A variant, expressed in the baculovirus system, thus bound both pentasaccharide and full-length heparin ∼5-fold more tightly than the K125M/rWT-III variant, expressed in the baby hamster kidney cell system. This affinity difference is presumably mainly due to the larger and more negatively charged carbohydrate side chains on the latter variant decreasing the affinity (*26*, *41*). A comparable difference in K_d between the N135A and rWT-III control variants was probably obscured by the uncertainties in the extrapolated K_d values for these variants but is indicated by the different elution positions of the two variants during heparin affinity chromatography (see also ref *43*). It was also apparent that the K125M/N135A variant bound both pentasaccharide and full-length heparin ∼2-fold more tightly than the similarly expressed and glycosylated K125Q/N135A variant.

Table 1: Dissociation Equilibrium Constants, Bimolecular Association Rate Constants, and Dissociation Rate Constants for Pentasaccharide and Full-Length Heparin Binding to the N135A, K125M/N135A, K125Q/N135A, rWT-III, N135Q, and K125M/rWT-III Antithrombin Variants at 25 °C, pH 7.4, $I = 0.15$[a]

heparin form	antithrombin variant	K_d (nM)	k_{on} ($\times 10^6$ M^{-1} s^{-1})	k_{off} (s^{-1})	calcd K_d (nM)[b]	calcd k_{off} (s^{-1})[c]
H5	N135A	~2[d,e]	70 ± 2[e]	nd[f]		~0.14[e]
	K125M/N135A	290 ± 50	4.9 ± 0.2	2.8 ± 0.2	570 ± 60	1.4 ± 0.3
	K125Q/N135A	720 ± 160	4.4 ± 0.5	6.0 ± 0.5	1400 ± 300	3.2 ± 1.0
	rWT-III	~2.5[d]	nd[f]	nd[f]		
	N135Q	~2[d]	50 ± 2	nd[f]		~0.1
	K125M/rWT-III	1400 ± 100	1.9 ± 0.2	12.4 ± 0.4	6500 ± 900	2.7 ± 0.5
H26	N135A	~0.15[d,e]	154 ± 1[e]	nd[f]		~0.02[e]
	K125M/N135A	50 ± 8	9.0 ± 0.2	0.8 ± 0.1	90 ± 20	0.4 ± 0.07
	K125Q/N135A	100 ± 10	11.5 ± 0.1	1.3 ± 0.1	110 ± 10	1.1 ± 0.1
	N135Q	~0.7[d]	81 ± 2	nd[f]		~0.08
	K125M/rWT-III	260 ± 10	3.3 ± 0.1	4.4 ± 0.3	1300 ± 130	0.86 ± 0.06

[a] Measured K_d values are averages ± SEM of at least three fluorescence titrations, except for pentasaccharide binding to the K125M/N135A variant and full-length heparin binding to the K125M/rWT-III variant, in which cases averages ± a range of two titrations are given. Measured values of k_{on} and k_{off} ± SEM were obtained by linear regression of plots of k_{obs} vs heparin concentration, comprising 5−7 points in the 0.2−1.2 μM range for binding of both saccharides to the N135A, K125M/N135A, K125Q/N135A, and N135Q variants and 7−15 points in the 0.2−8 and 0.8−4 μM ranges for binding of the pentasaccharide and full-length heparin, respectively, to the K125M/rWT-III variant. [b] From k_{on} and k_{off}. [c] From k_{on} and K_d. [d] Obtained by linear extrapolation of values measured at higher ionic strengths (see Figure 2A and similar data, not shown, for full-length heparin binding). [e] Taken from ref *22*. [f] Not determined.

Table 2: Ionic and Nonionic Contributions to Pentasaccharide Binding to the N135A, K125Q/N135A, rWT-III, N135Q, and K125M/rWT-III Antithrombin Variants at 25 °C and pH 7.4[a]

antithrombin form	Z	log(K_d')	K_d' (μM)
N135A	5.3 ± 0.3[b]	−5.1 ± 0.1[b]	7.9
K125Q/N135A	2.5 ± 0.1	−4.4 ± 0.1	40
rWT-III	5.2 ± 0.3	−5.0 ± 0.1	10
N135Q	5.0 ± 0.4	−5.2 ± 0.1	6.3
K125M/rWT-III	2.2 ± 0.1	−4.3 ± 0.1	50

[a] The number of ionic interactions (Z) involved in pentasaccharide binding to the antithrombin variants and the nonionic contribution [log(K_d')] to binding were determined from the slopes and intercepts, respectively, of plots of log(K_d) vs log[Na^+] (Figure 2A), as described previously (*12*, *26*, *37*). Errors represent ± SEM obtained by linear regression. [b] Taken from ref *22*.

Ionic and Nonionic Contributions to Pentasaccharide Binding. The ionic and nonionic contributions to the binding of the pentasaccharide to the K125Q/N135A and K125M/rWT-III antithrombin variants and to the three controls were evaluated from the dependence of dissociation equilibrium constants, measured by fluorescence titrations, on sodium ion concentration at pH 7.4. Log(K_d) varied linearly with log[Na^+] for the interaction of the pentasaccharide with all antithrombin forms that were studied (Figure 2A). According to polyelectrolyte theory (*12*, *26*, *37*, *44*), Z, the number of ionic interactions involved in the binding, can be obtained from the slope of such plots. Correspondingly, the intercept on the ordinate gives log(K_d'), the logarithm of the dissociation equilibrium constant at 1 M Na^+, reflecting the affinity of the nonionic interactions. All three control antithrombins made approximately five ionic interactions with the pentasaccharide, whereas both the K125Q/N135A and K125M/rWT-III variants only made approximately two such interactions (Table 2). Similarly, log(K_d') was approximately −5 for the control forms but was increased to approximately −4.3 for the two K125 mutants. Mutation of Lys125 thus caused both a loss of approximately three ionic interactions and an ~5-fold decrease in the affinity of the nonionic interactions. The similar data for the rWT-III and N135Q antithrombins again demonstrate the functional equivalence of these forms.

Rapid Kinetics Studies of Pentasaccharide and Full-Length Heparin Binding. The kinetics of binding of pentasaccharide and full-length heparin to the antithrombin variants were analyzed under pseudo-first-order conditions by stopped-flow fluorimetry at pH 7.4 and $I = 0.15$. The fluorescence increase was monophasic for all interactions and could be well fitted to a single-exponential function, giving the observed pseudo-first-order rate constant, k_{obs}. The overall association and dissociation rate constants, k_{on} and k_{off}, respectively, for the binding of both saccharides to the K125 mutants were obtained from the slope and intercept, respectively, of the linear increase in k_{obs} with saccharide concentration in the low-concentration range (Table 1). Accurate values of k_{on} for the control antithrombins could be determined in the same manner, whereas the k_{off} values for these forms were too small to be measured and were instead calculated from k_{on} and the estimated K_d (Table 1). All Lys125 mutations caused an appreciable (15−25-fold) decrease in k_{on} for the binding of both pentasaccharide and full-length heparin and an even greater increase in the observed k_{off}. However, the K_d calculated from k_{on} and k_{off} for the K125 variants was up to 5-fold higher than the measured K_d, probably because of an anomalously high observed k_{off}. As found previously for other antithrombin mutants (*22*−*24*), such a discrepancy between calculated and measured values of K_d is thus most likely due to a small contribution of a preequilibrium pathway, in addition to the predominant induced-fit pathway, for heparin activation of the K125 variants. In this preequilibrium pathway, heparin binds preferentially to a small amount of already conformationally activated antithrombin in equilibrium with the unactivated inhibitor (*36*). Also, a minor contribution of this pathway may result in an anomalously high k_{off} being measured without k_{on} being appreciably affected (*22*). The values of k_{off} calculated from K_d and k_{on} (Table 1) therefore presumably are more accurate than the measured ones. Comparisons of these values with those for the controls show that the Lys125 mutations nevertheless caused a substantial (10−50-fold) increase in k_{off} for the binding of both saccharides, the variation in the magnitude of the increase most likely being to a large extent due to the approximate control values. These kinetic analyses also show

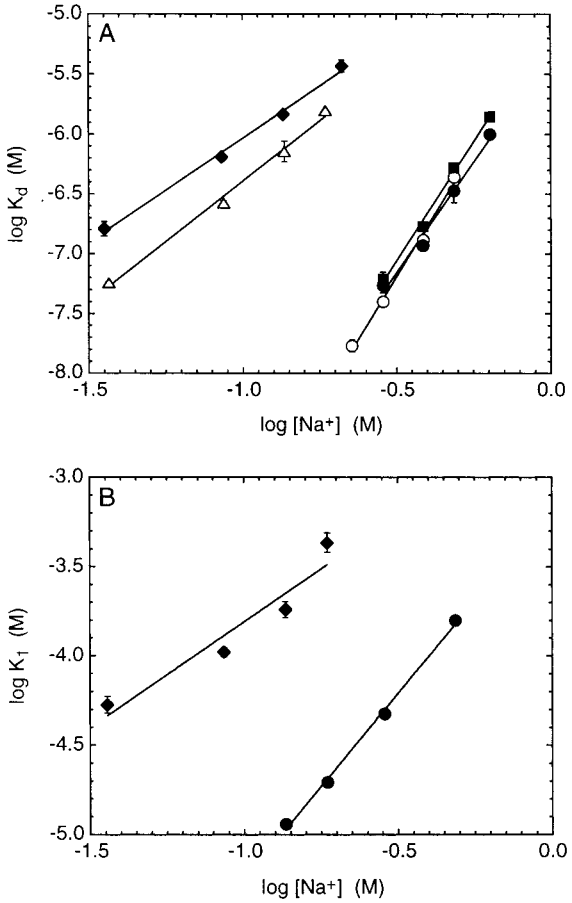

FIGURE 2: Sodium ion concentration dependence of dissociation equilibrium constants for pentasaccharide binding to the antithrombin variants at 25 °C and pH 7.4. (A) Overall dissociation constants for binding to the N135A, K125Q/N135A, rWT-III, N135Q, and K125M/rWT-III variants and (B) dissociation constants for the first step of binding to the N135Q and K125M/rWT-III variants: (○) N135A, (△) K125Q/N135A, (■) rWT-III, (●) N135Q, and (◆) K125M/rWT-III. Values are averages ± the standard error of the mean (SEM) derived from at least three determinations. Error bars not shown lie within the dimensions of the symbols. The solid lines represent linear regression fits. The data for the N135A variant are from previous work (*22*).

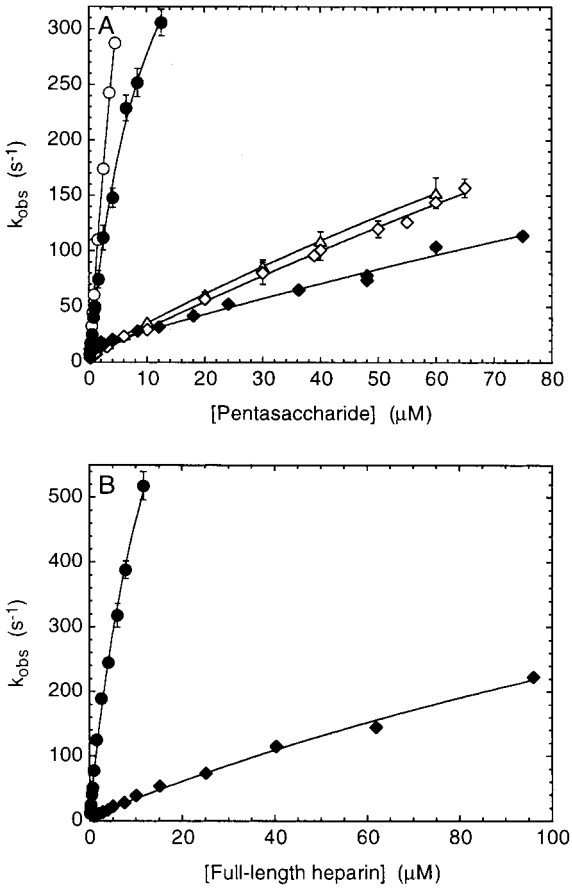

FIGURE 3: Heparin concentration dependence of observed pseudo-first-order rate constants for pentasaccharide and full-length heparin binding to the antithrombin variants at 25 °C, pH 7.4, and I = 0.15. (A) Pentasaccharide and (B) full-length heparin: (○) N135A, (◇) K125M/N135A, (△) K125Q/N135A, (●) N135Q, and (◆) K125M/rWT-III. Values are averages ± SEM derived from 8−16 individual measurements. Error bars not shown lie within the dimensions of the symbols. The solid lines represent nonlinear regression fits to eq 1. The data for the N135A variant are from previous work (*22*), in which the analyses were extended to a k_{obs} of ∼400 s^{-1}, revealing the hyperbolic concentration dependence.

that the higher pentasaccharide and full-length heparin affinity of the K125M/N135A variant compared with that of the more extensively glycosylated K125M/rWT-III variant was due to both a 2−3-fold higher k_{on} and a similarly lower k_{off}. A comparable difference in k_{on} was also apparent for the N135A and N135Q variants, whereas the uncertainties in k_{off} precluded any comparisons of this parameter between the two control forms. By contrast, the tighter binding of the two saccharides by the K125M/N135A variant than by the K125Q/N135A variant was caused predominantly by an ∼2-fold lower k_{off}.

Kinetic analyses of the binding of the pentasaccharide and full-length heparin to the K125 mutants extended to high concentrations of the two saccharides showed a hyperbolic dependence of k_{obs} on saccharide concentration (Figure 3). This behavior indicates that the two heparin forms bind to the mutants by the two-step, induced-fit mechanism previously defined for heparin binding to the plasma antithrombin α and β isoforms, recombinant wild-type and N135A antithrombin and other mutants of the inhibitor affecting heparin binding (*11*, *12*, *22*−*24*, *26*). This mechanism involves a weak initial binding of heparin in a rapid equilibrium, followed by a conformational change of antithrombin that is responsible for the observed fluorescence change, tightens the binding, and activates the inhibitor (Scheme 1).

Scheme 1

$$AT + H \overset{K_1}{\rightleftharpoons} AT{-}H \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} AT^*{-}H$$

In this scheme, AT is antithrombin, H is heparin, AT−H is the initial weak complex, AT*−H is the final, conformationally altered complex, K_1 is the dissociation equilibrium constant of the first, weak binding step, and k_{+2} and k_{-2} are the forward and reverse rate constants, respectively, of the second, conformational change step. This two-step binding mechanism leads to k_{obs} increasing hyperbolically with the total heparin concentration, $[H]_o$, according to the equation (*11*, *12*)

$$k_{obs} = \frac{k_{+2}[H]_o}{[H]_o + K_1} + k_{-2} \qquad (1)$$

Table 3: Kinetic Constants for the Two-Step Mechanism of Pentasaccharide and Full-Length Heparin Binding to the N135A, K125M/N135A, K125Q/N135A, N135Q, and K125M/rWT-III Antithrombin Variants at 25 °C, pH 7.4, and $I = 0.15$[a]

heparin form	antithrombin variant	K_1 (μM)	k_{+2} (s^{-1})	k_{-2} (s^{-1})	k_{+2}/k_{-2}
H5	N135A	28 ± 4[b]	2100 ± 300[b]	~0.14	~15000
	K125M/N135A	340 ± 110	950 ± 270	1.4 ± 0.3	680
	K125Q/N135A	290 ± 50	860 ± 140	3.2 ± 1.0	270
	N135Q	11 ± 1	570 ± 40	~0.1	~5700
	K125M/rWT-III	190 ± 70	350 ± 110	2.7 ± 0.5	130
H26	N135Q	10 ± 1	890 ± 80	~0.08	~11000
	K125M/rWT-III	130 ± 30	460 ± 90	0.86 ± 0.06	530

[a] The dissociation equilibrium constant of the first step, K_1, and the forward rate constant of the second step, k_{+2}, of the two-step binding mechanism in Scheme 1 were obtained by nonlinear regression fits of the data in Figure 3 to eq 1. The reverse rate constant of the second step, k_{-2}, of this mechanism was taken from Table 1 as the calculated value of k_{off}. Errors represent ±SEM. [b] Taken from ref *22*.

Moreover, in this mechanism, k_{on} and k_{off}, measured at low saccharide concentrations, are equal to k_{+2}/K_1 and k_{-2}, respectively (*12*). Values of K_1 and k_{+2} for pentasaccharide binding to the three K125 variants and the N135A and N135Q controls, as well as for full-length heparin binding to the K125M/rWT-III variant and the N135Q control, were derived by nonlinear regression fits to eq 1 of the hyperbolic dependence of k_{obs} on saccharide concentration (Figure 3 and Table 3). Corresponding kinetic analyses of the binding of full-length heparin to the K125M/N135A and K125Q/N135A variants were not done, as K_1 and k_{+2} for such binding to the N135A control variant cannot be properly quantified by measurements within the time frame accessible by stopped-flow kinetics (*22*, *26*). The K125 mutations resulted in an appreciable (10−17-fold) increase in K_1 for both pentasaccharide and full-length heparin binding at $I = 0.15$. A comparable ~12-fold increase in K_1 was also observed in analyses of pentasaccharide binding to the K125M/rWT-III and N135Q variants at $I = 0.05$ (not shown). At this ionic strength, K_1 for both variants is lower than at $I = 0.15$, allowing pentasaccharide concentrations around K_1 to be reached for the K125M/rWT-III variant. In contrast to the considerable effect on K_1, k_{+2} was only moderately (~2-fold) decreased for both pentasaccharide and full-length heparin binding to the K125 variants at $I = 0.15$, the increased K_1 thus accounting for most of the observed decrease in k_{on} caused by the substitutions. k_{-2}, being equal to k_{off} and taken as the calculated, presumably more accurate value of k_{off} (see above), was also substantially (10−50-fold) increased by the mutations for both saccharides at $I = 0.15$ (Table 3). As a consequence of the effects on k_{+2} and k_{-2}, the equilibrium constant for the second step of binding of the saccharides (k_{+2}/k_{-2}) is greatly decreased by the mutations, although the conformationally altered, activated state induced by the binding is still highly favored (Table 3). Comparisons of the data for the K125M/N135A and N135A variants with those for the corresponding, more extensively glycosylated K125M/rWT-III and N135Q variants show that the higher k_{on} of the former variants was caused primarily by an ~3-fold higher k_{+2}.

Nature of Interactions in the First Step of Pentasaccharide Binding. The nature of the interactions made by Lys125 in the first, weak step of pentasaccharide binding was elucidated by evaluation of the ionic strength dependence of the dissociation constant of this step, K_1, for the N135Q and K125M/rWT-III variants at pH 7.4. K_1 was obtained by measuring k_{on} for pentasaccharide binding to the two variants in the manner described above at different ionic strengths and deriving K_1 from the relationship $K_1 = k_{+2}/k_{on}$ (*12*, *26*). The values of k_{+2} determined at $I = 0.15$ were used at all ionic strengths in these calculations, a reasonable assumption based on previous studies showing that k_{+2} for pentasaccharide binding to plasma antithrombin is independent of ionic strength (*12*). Plots of $\log(K_1)$ versus $\log[Na^+]$ (Figure 2B) gave Z_1 and $K_d'_{,1}$, the number of charge interactions and the dissociation constant of the nonionic interactions, respectively, established in the first binding step. Values for Z_1 of 2.6 ± 0.1 and 1.5 ± 0.3 were obtained for the N135Q and K125M/rWT-III variants, respectively, indicating a loss of approximately one ionic interaction in the first step on mutation of Lys125. Correspondingly, $K_d'_{,1}$ was 0.7 ± 0.1 and 2.4 ± 0.8 mM for the control and mutant antithrombins, respectively, consistent with a 3−4-fold weakening of the nonionic interactions established in the first step as a consequence of the mutation.

Affinity of Binding of Saccharides Lacking the Reducing or Nonreducing Ends of the Pentasaccharide. The extent to which the ionic and nonionic interactions between Lys125 and the pentasaccharide are dependent on either end of the pentasaccharide was assessed by measuring the affinity of binding of three tetra- or trisaccharides to the K125M/rWT-III and rWT-III antithrombin variants. Two of these saccharides, DEFG* and DEF, lack one and two monosaccharide units, respectively, in the reducing end of the pentasaccharide, designated DEFGH, and thus represent the non-reducing-end tetra- and trisaccharide, respectively, of the latter (*36*). Correspondingly, the third saccharide, EFGH′, lacks the non-reducing-end monosaccharide unit and thus represents the reducing-end tetrasaccharide (*36*). The DEFG* and EFGH′ tetrasaccharides have slight structural modifications, compared with the pentasaccharide, in groups that are not essential for binding to antithrombin (*36*). We have previously shown that these oligosaccharides, although binding more weakly than the pentasaccharide, compete with the latter for binding to plasma antithrombin and introduce an activating conformational change in the inhibitor comparable to that of the pentasaccharide (*36*). The binding affinities were measured by fluorescence titrations at pH 6.0 and $I = 0.025$, these conditions being dictated by the affinity for the DEF trisaccharide being too low to be measurable at higher pH and ionic strength. In general, the mutant and wild-type antithrombins bound the saccharides lacking residues in either end of the pentasaccharide more weakly than the intact pentasaccharide (Table 4), but the reductions in affinity were different for the two antithrombin forms. The ratio between the dissociation constants for binding of a saccharide to the

Table 4: Dissociation Equilibrium Constants for Binding of the Pentasaccharide and Tetra- or Trisaccharides Lacking the Reducing or Nonreducing Ends of the Pentasaccharide to the rWT-III and K125M/rWT-III Antithrombin Variants at 25 °C, pH 6.0, and $I = 0.025$[a]

saccharide	rWT-III (μM)	K125M/rWT-III (μM)	$K_{d,rWT-III}/K_{d,K125M}$
DEFGH	$\sim 3 \times 10^{-8}$[b]	~ 0.006[b]	~ 200000
DEFG*	~ 0.003[b]	3.4 ± 0.2	~ 1100
DEF	0.26 ± 0.01	140 ± 30	540
EFGH′	0.21 ± 0.02	2.6 ± 0.2	12

[a] Measured K_d values are averages ± a range of two fluorescence titrations. DEFGH designates the normal pentasaccharide, the letters indicating the individual monosaccharide units from the nonreducing end. Tetrasaccharide DEFG* and trisaccharide DEF lack monosaccharide unit H and disaccharide unit GH, respectively, in the reducing end of the pentasaccharide, whereas tetrasaccharide EFGH′ lacks the non-reducing-end monosaccharide unit D. The asterisk and prime notations denote modifications in groups not essential for binding (*36*). The full structures of the saccharides are given in ref *36*. [b] Obtained by linear extrapolation of values measured at higher ionic strengths, i.e., from data, not shown, similar to those in Figure 2A.

control and to the K125 mutant ($K_{d,rWT-III}/K_{d,K125M}$) is a measure of the defect in binding of that particular saccharide introduced by the K125 mutation. A reduction of this ratio for a saccharide lacking one or more residues from that for the pentasaccharide thus indicates the extent to which the interactions between the pentasaccharide and Lys125 are dependent on the missing saccharide residue or residues. The $K_{d,rWT-III}/K_{d,K125M}$ ratio was reduced from ~200000 for the pentasaccharide to ~10 for the EFGH′ tetrasaccharide (Table 4), indicating that the non-reducing-end residue D is of major importance for the interactions between the pentasaccharide and Lys125. The reduction of this ratio was much smaller, to ~1000 and ~500 for the DEFG* and DEF saccharides, respectively, consistent with the reducing-end residues G and H being appreciably less important for these interactions.

Kinetics of Proteinase Inhibition. Second-order rate constants for the uncatalyzed and pentasaccharide- or full-length heparin-catalyzed inhibition of thrombin or factor Xa by the K125M/N135A, K125Q/N135A, and K125M/rWT-III antithrombin variants and their respective controls were determined by discontinuous assays of residual proteinase activity at pH 7.4 and $I = 0.15$ (Table 5). The pentasaccharide enhancement of the rate of thrombin inhibition, being less than 2-fold for wild-type antithrombin (*12*), was not investigated. Similarly, the effect of full-length heparin on the rate of inactivation of factor Xa was not evaluated in case of the K125M/rWT-III variant, as the pentasaccharide accounts for nearly full enhancement of the rate of inactivation of this proteinase by the wild-type inhibitor (*12*, *43*) or the N135A variant (Table 5). All uncatalyzed rate constants, as well as the catalyzed rate constants, i.e., the rate constants at saturation of antithrombin with the saccharides, for the inhibition of the two proteinases by the three Lys125 mutants were essentially indistinguishable from those of the corresponding control antithrombins. The somewhat higher rate constant for pentasaccharide-catalyzed inhibition of factor Xa by the K125M/rWT-III variant, compared with the values for the rWT-III and N135Q controls, is presumably a consequence of an appreciable error in calculating the concentration of the complex between the pentasaccharide and this variant, due to the weak binding. The similar rate constants measured for the rWT-III and N135Q controls provide further evidence that these two antithrombin forms have equivalent functional properties.

Table 5: Association Rate Constants for Uncatalyzed and Pentasaccharide- or Full-Length Heparin-Catalyzed Inhibition of Proteinases by the N135A, K125M/N135A, K125Q/N135A, rWT-III, N135Q, and K125M/rWT-III Antithrombin Variants at 25 °C, pH 7.4, and $I = 0.15$[a]

proteinase	antithrombin variant	k_{uncat} ($\times 10^3$ M^{-1} s^{-1})	k_{H5} ($\times 10^5$ M^{-1} s^{-1})	k_{H26} ($\times 10^6$ M^{-1} s^{-1})
thrombin	N135A	9.4 ± 0.4[b]	nd[c]	9.0 ± 0.5[b]
	K125M/N135A	10.9 ± 0.9	nd[c]	7.3 ± 0.3
	K125Q/N135A	11.4 ± 0.7	nd[c]	7.3 ± 1.0
	rWT-III	10.0 ± 0.5	nd[c]	14.5 ± 3.2
	N135Q	9.2 ± 0.4[d]	nd[c]	13.0 ± 1.0[d]
	K125M/rWT-III	8.0 ± 1.5	nd[c]	10.4 ± 3.2
factor Xa	N135A	4.8 ± 0.2[b]	6.1 ± 0.1[b]	1.2 ± 0.04[b]
	K125M/N135A	6.3 ± 0.3	7.1 ± 0.4	1.4 ± 0.1
	K125Q/N135A	6.1 ± 0.5	6.8 ± 1.2	1.4 ± 0.1
	rWT-III	4.2 ± 0.5	3.7 ± 0.4	nd[c]
	N135Q	4.5 ± 0.1[d]	4.6 ± 0.6[d]	nd[c]
	K125M/rWT-III	4.3 ± 0.1	9.1 ± 0.2	nd[c]

[a] Second-order association rate constants for uncatalyzed (k_{uncat}), pentasaccharide-catalyzed (k_{H5}), and full-length heparin-catalyzed (k_{H26}) reactions of the antithrombin variants with the proteinases were determined as described in Materials and Methods. Uncatalyzed rate constants are averages ± SEM of at least three measurements. Most values of k_{H5} and k_{H26} ± SEM were obtained by linear regression of plots of k_{obs} vs heparin concentration, comprising at least three points in the 0.1−10 nM range. The value of k_{H5} for the inhibition of factor Xa by the K125M/rWT-III variant is the average ± SEM of three determinations at two separate pentasaccharide concentrations. Uncatalyzed rate constants for inhibition of thrombin were unaffected by 50 μg/mL Polybrene, verifying that the antithrombin preparations were not contaminated by heparin. [b] Taken from ref *22*. [c] Not determined. [d] Taken from ref *30*.

DISCUSSION

The importance of Lys125 of antithrombin for heparin binding was first indicated by chemical modification (*16*) and was later substantiated by a site-directed mutagenesis study that showed an appreciable 30−150-fold loss in heparin binding affinity on mutation of Lys125 to Met (*18*). In contrast, only an ~2-fold heparin binding defect was reported for a Lys125 to Gln antithrombin mutant (*19*). Alanine scanning mutagenesis of several residues in the heparin-binding region also supported an essential role of Lys125 in the binding, although the decrease in affinity was not quantified (*21*). In agreement with Lys125 being important for heparin binding, the X-ray structure of the complex between antithrombin and a heparin pentasaccharide indicates that Lys125 makes close contacts with negatively charged groups in the non-reducing-end disaccharide unit of the pentasaccharide (Figure 1) (*14*). Whereas this structure thus clearly implicates Lys125 in heparin binding, it gives no information about the relative importance of this residue for the binding or for the activation of the inhibitor. Studies of the effects of site-directed mutagenesis of Lys125 on the affinity and kinetics of heparin binding are necessary in obtaining such information.

In this work, substitution of Lys125 with Met or Gln was shown to result in 150−600-fold decreases in affinity for both pentasaccharide and full-length heparin at pH 7.4, $I = 0.15$, and 25 °C, with only small differences between the three variants that were studied. These reductions are

somewhat higher than those reported previously for a similar K125M mutant under the same conditions (*18*), apparently due to difficulties in quantifying the tight binding to the control in the previous work. Such difficulties were overcome in this work by extrapolating more accurately determined values of K_d at higher ionic strengths to $I = 0.15$. The affinity losses observed in this study correspond to 12−16 kJ/mol in free energy of binding, i.e., 25−33% of the total binding free energy. This decrease in binding energy is comparable to that found previously for mutation of Arg129 (*23*) but ∼2-fold larger than and half of the decreases shown for mutations of Arg47 and Lys114, respectively (*22*, *24*). The Lys125 mutations did not affect the uncatalyzed rates of antithrombin inhibition of thrombin or factor Xa or the ability of the pentasaccharide or full-length heparin to normally accelerate antithrombin inhibition of the two proteinases at saturation of the inhibitor with the saccharides. Moreover, they did not affect the ability of the two saccharides to induce protein fluorescence changes indicative of normal conformational activation of antithrombin. These observations provide strong evidence that the mutations affected only the heparin binding affinity but not the native or activated conformations of antithrombin. Together, the results therefore show that Lys125 is a major heparin-binding residue of antithrombin, contributing more binding energy than Arg47, an amount of energy comparable to that of Arg129, but less energy than Lys114.

The reduction in antithrombin affinity for pentasaccharide and full-length heparin on mutation of Lys125 was shown to arise both from disruption of approximately three ionic interactions and from weakened nonionic interactions. The losses of the two types of binding accounted for ∼75 and ∼25%, respectively, of the total decrease in binding energy. The finding that elimination of the single positive charge on Lys125 causes three ionic interactions to be broken suggests that the charge interaction involving Lys125 is necessary for two other comparable charge interactions to be established. Previous work has shown that mutation of Lys114 and Arg129, but not of Arg47, similarly leads to the loss of multiple ionic interactions between antithrombin and heparin (*22*−*24*). Lys114, Lys125, and Arg129 therefore presumably constitute the core of a cooperative network of residues interacting with the heparin pentasaccharide region. This network contributes not only ionic but also appreciable nonionic interactions, i.e., hydrogen bonds, hydrophobic interactions, or van der Waals interactions, between antithrombin and the saccharide. The importance of the three key basic residues for the cooperativity may primarily be due to these residues, in particular, Lys114 (*24*), playing pivotal roles in the conformational changes involved in antithrombin activation.

Rapid kinetics studies showed that both pentasaccharide and full-length heparin bound to the Lys125 antithrombin mutants by the same two-step mechanism (Scheme 1) previously established for plasma and recombinant antithrombins (*11*, *12*, *26*). However, the Lys substitutions caused an appreciable, 10−17-fold, decrease in the affinity of the initial, weak binding step of this mechanism for both saccharides. This effect is in contrast to those of substitutions of Arg47, Lys114, and Arg129, which all minimally affected the affinity of the first step (*22*−*24*). Lys125 is thus the first antithrombin residue to be identified that contributes a significant amount of binding energy (∼6−7 kJ/mol) to the initial recognition of heparin. The affinity contributed by Lys125 to the first heparin binding step originates both from approximately one ionic interaction, as expected, and from nonionic interactions. The two interaction types comprise ∼70 and ∼30%, respectively, of the total free energy of the initial binding step, thus mirroring the nature of the overall contributions to heparin binding made by Lys125. It is noteworthy that no appreciable reduction in the affinity of the first heparin binding step was observed on mutation of either Lys114 or Arg129, although these residues appear to be involved in a cooperative network that includes Lys125. This cooperative coupling is therefore presumably not established until the second, conformational change step of heparin binding by a reorganization of ionic interactions that leads to the final, tight binding.

In addition to increasing the affinity of the first step of heparin binding to antithrombin, Lys125 is also important for the second step. Mutation of Lys125 thus moderately, ∼2-fold, decreased the forward rate constant and appreciably, 10−50-fold, increased the reverse rate constant of this step. The role of Lys125 in the second heparin binding step is therefore mainly in stabilizing the conformationally activated state of antithrombin but less in promoting the rate at which this state is attained. In both these respects, Lys125 plays a role quantitatively comparable to that of Arg47 but less prominent than those of Arg129 and, in particular, Lys114 (*22*−*24*). Despite the effects of the Lys125 mutation on both rate constants of the conformational change step, the equilibrium of this step remained strongly shifted toward the activated conformation. This favorable equilibrium accounts for the observed normal rate of proteinase inactivation by the mutated inhibitor on saturation with pentasaccharide or full-length heparin.

Recent studies of the binding to plasma antithrombin of saccharides lacking units in the nonreducing or reducing ends of the heparin pentasaccharide have suggested a two-site, allosteric model for pentasaccharide binding, in which the two ends play different roles (*36*, *45*). In this model, only the rigid, non-reducing-end trisaccharide unit, designated DEF (Figure 1), of the pentasaccharide, designated DEFGH, interacts with antithrombin in the first step. The conformational change in the inhibitor induced by this initial binding leads both to tighter interactions with the DEF unit and to new interactions with the reducing-end disaccharide unit, GH, of the pentasaccharide. These latter interactions thus serve mainly to lock the inhibitor in the conformationally activated state. The results of the work presented here are in agreement with this model. The finding that Lys125 is of major importance for the initial heparin binding step is thus consistent with this residue interacting with the D and E saccharides in the X-ray structure of the antithrombin−pentasaccharide complex (Figure 1) (*14*). Such an interaction is further supported by the studies of binding of saccharides lacking units in either of the two ends of the pentasaccharide to the K125M mutant in this work. The role of Lys125 in the initial binding is presumably related to its location in such a position that only a minor conformational reorientation is necessary for it to form contacts with the appropriate groups on the pentasaccharide (Figure 1). The demonstrated contributions of Lys125 to increasing the rate of the conformational change induced by the initial binding and to

decreasing the rate of reversal of this change are also consistent with its interactions with the non-reducing-end D and E saccharides of the pentasaccharide. Moreover, it is likely that those interactions that are coupled to Lys125 in the cooperative network similarly involve primarily the nonreducing end of the pentasaccharide.

In conclusion, Lys125 of antithrombin is of appreciable importance for heparin binding, contributing an amount of binding energy comparable to that of Arg129 but less energy than Lys114. Like these two residues, it is a key member of a network of positively charged side chains that cooperatively interact with the polysaccharide. Lys125 is the first residue of antithrombin shown to be of importance for the initial, weak binding step of the two-step heparin binding mechanism. In addition, it moderately increases the rate of induction of the conformational change of antithrombin in the second step and appreciably contributes to keeping the inhibitor in the conformationally activated state. These effects are all results of interactions with the two non-reducing-end saccharides of the heparin pentasaccharide.

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