The Region of Antithrombin Interacting with Full-Length Heparin Chains Outside the High-Affinity Pentasaccharide Sequence Extends to Lys136 but Not to Lys139[†]

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ABSTRACT: The interaction of a well-defined pentasaccharide sequence of heparin with a specific binding site on antithrombin activates the inhibitor through a conformational change. This change increases the rate of antithrombin inhibition of factor Xa, whereas acceleration of thrombin inhibition requires binding of both inhibitor and proteinase to the same heparin chain. An extended heparin binding site of antithrombin outside the specific pentasaccharide site has been proposed to account for the higher affinity of the inhibitor for full-length heparin chains by interacting with saccharides adjacent to the pentasaccharide sequence. To resolve conflicting evidence regarding the roles of Lys136 and Lys139 in this extended site, we have mutated the two residues to Ala or Gln. Mutation of Lys136 decreased the antithrombin affinity for full-length heparin by at least 5-fold but minimally altered the affinity for the pentasaccharide. As a result, the full-length heparin and pentasaccharide affinities were comparable. The reduced affinity for full-length heparin was associated with the loss of one ionic interaction and was caused by both a lower overall association rate constant and a higher overall dissociation rate constant. In contrast, mutation of Lys139 affected neither full-length heparin nor pentasaccharide affinity. The rate constants for inhibition of thrombin and factor Xa by the complexes between antithrombin and full-length heparin or pentasaccharide were unaffected by both mutations, indicating that neither Lys136 nor Lys139 is involved in heparin activation of the inhibitor. Together, these results show that Lys136 forms part of the extended heparin binding site of antithrombin that participates in the binding of full-length heparin chains, whereas Lys139 is located outside this site.

Antithrombin is a plasma serpin that plays a major role in the regulation of blood clotting, primarily by inhibiting the coagulation serine proteinases thrombin and factor Xa (*1*, *2*). Like other inhibitory serpins, antithrombin inactivates its target proteinases by irreversibly forming stable equimolar complexes in which the proteinase active site is nonfunctional. Antithrombin inhibition of clotting proteinases is slow but is accelerated up to several thousandfold by the anticoagulant sulfated polysaccharide heparin (*2*). Anticoagulantly active heparin species bind to antithrombin with a dissociation constant of 10−20 nM at physiological ionic strength and pH through a specific pentasaccharide sequence (*3*, *4*). The binding is a two-step process, in which an initial weak interaction between heparin and antithrombin induces a conformational change of the inhibitor (*4*, *5*). This change leads to tighter heparin binding and increases antithrombin reactivity toward factor Xa. The conformational change in antithrombin induced by the specific pentasaccharide accounts for most of the accelerating effect of heparin on the inhibition of factor Xa but is insufficient for accelerating thrombin inhibition (*4*). Instead, the latter effect requires a longer heparin molecule of at least 18 monosaccharide units (*6*, *7*) that can bind both proteinase and inhibitor and thus accelerate the reaction by a bridging mechanism.

Binding of heparin to antithrombin involves ionic interactions between negatively charged groups on the polyanionic heparin and positively charged amino acid residues on antithrombin. Appreciable nonionic interactions are also established, consistent with the specificity of the binding (*7*). A number of basic residues, including Lys11, Arg13, Arg46, Arg47, Lys114, Lys125, and Arg129, have been proposed to participate in pentasaccharide binding by studies of chemically modified or mutated antithrombins, as well as by the recent crystal structure of antithrombin in complex

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with a heparin pentasaccharide (*8*−*18*). In addition, several other basic residues, localized outside the pentasaccharide binding site, viz., Arg132, Lys133, Lys136, and Lys139, have been suggested to bind to saccharide units adjacent to the pentasaccharide sequence in longer heparin chains (*11*, *14*, *15*, *19*). This binding most likely accounts for the increased antithrombin affinity for such chains but does not appear to be involved in the conformational change that activates the inhibitor (*4*, *20*). However, the roles of Lys136 and Lys139 in this binding are unclear. Lys136 was originally implicated in heparin binding by chemical modification (*11*), but a recombinant antithrombin variant with a mutation of this residue was reported to have a normal heparin affinity (*14*). Moreover, the crystal structure of the pentasaccharide−antithrombin complex suggests that a contribution of Lys139 to binding of longer heparin chains, as was proposed from studies of a recombinant antithrombin variant (*14*, *15*), is unlikely (*17*).

To further investigate the roles of lysines 136 and 139 in heparin binding to and activation of antithrombin, we have individually mutated these residues in a recombinant antithrombin, expressed in a baculovirus system. Mutation of Lys136 reduced the affinity of antithrombin for full-length heparin by at least 5-fold but resulted in only a minor decrease in pentasaccharide affinity. In contrast, mutation of Lys139 did not affect the affinity for either full-length heparin or pentasaccharide. The ionic strength dependence of the dissociation equilibrium constant for full-length heparin binding showed that the decrease in affinity caused by the Lys136 mutation was due to the loss of one ionic interaction. The rates of inhibition of thrombin and factor Xa by either variant, both alone and in complex with heparin, were normal, indicating that the mutations did not affect the native antithrombin conformation and that neither Lys136 nor Lys139 is involved in the mechanism of heparin activation of the inhibitor. Together, these results show that the region outside the high-affinity pentasaccharide binding site of antithrombin that participates in binding of full-length heparin chains extends to Lys136 but does not involve Lys139.

MATERIALS AND METHODS

Proteins. Recombinant antithrombin variants were produced and expressed in a baculovirus system (*16*, *18*, *21*). The S137T[1] and N135A antithrombin variants characterized previously (*20*−*22*) were used as base molecules in this study. The S137T substitution induces complete glycosylation of the Asn135−Ser137 glycosylation site (*22*), whereas the N135A substitution prevents glycosylation of this site, producing variants similar to the α- and *β*-forms of plasma antithrombin, respectively (*23*, *24*). The K136A/N135A and K139A/N135A variants characterized in this work were produced by additional Lys136 and Lys139 substitutions on the *β* background and the K139Q/S137T variant by an additional Lys139 to Gln substitution on the α background. The antithrombin variants were purified by affinity chromatography on immobilized heparin (*16*, *18*, *20*, *21*).

The antithrombin preparations were analyzed by SDS−PAGE and nondenaturing electrophoresis with the Tricine and Laemmli buffer systems, respectively (*25*, *26*), and by rechromatography on a 1 mL HiTrap Heparin (Amersham Pharmacia Biotech, Uppsala, Sweden) column (*18*). Protein concentrations of the variants were determined from absorbance measurements at 280 nm with the use of a molar extinction coefficient of 37 700 M^{-1} cm^{-1} (*27*).

Heparins. Full-length heparin with high affinity for antithrombin and with an average molecular weight of ∼8000 and reduced polydispersity was prepared as described earlier (*4*, *7*, *28*). The antithrombin-binding heparin pentasaccharide (*29*) was a gift from Dr. M. Petitou (Sanofi Recherche, Toulouse, France). Concentrations of both saccharide forms were determined by stoichiometric fluorescence titrations with plasma antithrombin (*4*).

Experimental Conditions. All measurements were done at 25.0 ± 0.2 °C in 20 mM sodium phosphate buffer, pH 7.4, containing 100 *μ*M EDTA and 0.1% (w/v) poly(ethylene glycol) 8000. NaCl was added to ionic strengths of 0.15−0.6.

Fluorescence Titrations. Stoichiometries of full-length heparin binding and affinities of full-length heparin or pentasaccharide binding to the antithrombin variants were measured by titrations monitored by the increase in tryptophan fluorescence that accompanies the interaction, as described previously (*4*, *7*, *18*, *20*). The results were analyzed by nonlinear least-squares fitting of the data to the equilibrium binding equation (*3*, *5*, *28*).

Kinetics of Heparin Binding. The rates of binding of full-length heparin or pentasaccharide to the antithrombin variants were measured under pseudo-first-order conditions by stopped-flow fluorometry, as in earlier work (*4*, *18*, *20*). Values of k_{obs} were obtained by nonlinear regression fitting of the progress curves to a single exponential function. Four traces were typically averaged for each rate constant determination, and reported k_{obs} values are averages of at least 4 such determinations.

Stoichiometries and Kinetics of Proteinase Inactivation. Stoichiometries of inhibition of active-site-titrated human α-thrombin (a gift from Dr. John Fenton, New York State Department of Health, Albany, NY) by the antithrombin variants were determined as detailed previously (*18*). Second-order rate constants for inhibition of human α-thrombin or factor Xa (*30*) by the variants alone and in complex with full-length heparin or pentasaccharide were measured under pseudo-first-order conditions at ionic strength 0.15, as in earlier work (*18*, *20*, *31*). Observed pseudo-first-order rate constants were obtained by fitting the data by nonlinear regression to a single-exponential decay function with an endpoint of zero activity (*28*). The second-order rate constants were obtained from these data as described previously (*31*).

The effect of the different affinities of the N135A and K136A/N135A variants for full-length heparin on the rate of proteinase inactivation in the presence of the polysaccharide was shown by incubating 10 nM antithrombin variant and 1 nM factor Xa with or without 10 nM heparin at ionic

[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; K136A, substitution of Lys136 by Ala; K139A, substitution of Lys139 by Ala; K139Q, substitution of Lys139 by Gln; N135A, substitution of Asn135 by Ala; S137T, substitution of Ser137 by Thr; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Table 1: Dissociation Equilibrium Constants, Bimolecular Association Rate Constants, and Dissociation Rate Constants for Full-Length Heparin and Pentasaccharide Binding to the N135A, K136A/N135A, K139A/N135A, S137T, and K139Q/S137T Antithrombin Variants at 25 °C, pH 7.4, Ionic Strengths 0.3 and 0.4[a]

ionic strength	heparin form	antithrombin variant	K_d (nM)	$10^{-6}\cdot k_{on}$ ($M^{-1}\cdot s^{-1}$)	k_{off} (s^{-1})	calculated K_d (nM)[b]
0.3	H26	N135A	7 ± 1	23 ± 0.2	0.3 ± 0.1	13 ± 5
		K136A/N135A	50 ± 1	10 ± 0.4	0.5 ± 0.2	50 ± 20
		K139A/N135A	6 ± 2	nd[c]	nd	
		S137T	80 ± 10	nd	nd	
		K139Q/S137T	110 ± 10	nd	nd	
	H5	N135A	40 ± 4	28 ± 1	1.5 ± 0.4	55 ± 15
		K136A/N135A	84 ± 3	24 ± 1	1.7 ± 0.4	70 ± 20
		S137T	340 ± 10	nd	nd	
		K139Q/S137T	420 ± 20	nd	nd	
0.4	H26	N135A	32 ± 2	9.8 ± 0.2	0.4 ± 0.1	40 ± 10
		K136A/N135A	170 ± 15	4.5 ± 0.05	0.76 ± 0.05	170 ± 10
	H5	N135A	130 ± 10	11.1 ± 0.2	1.7 ± 0.1	150 ± 10
		K136A/N135A	210 ± 2	9.5 ± 0.4	1.7 ± 0.3	180 ± 40

[a] The K_d values are averages ± SE of at least three fluorescence titrations. The k_{on} and k_{off} values ± SE were obtained by linear regression of plots of k_{obs} vs heparin concentration, comprising 5−6 points in the 0.05−0.9 μM concentration range. [b] From k_{on} and k_{off}. [c] nd, not determined.

strength 0.4. After different reaction times, the chromogenic substrate Spectrozyme FXa (American Diagnostica, Greenwich, CT) was added to a concentration of 100 μM, and the residual enzyme activity was determined by measuring the initial rate of substrate hydrolysis at 405 nm. The data were fitted by nonlinear regression as described above.

RESULTS

Expression and Homogeneity of Antithrombin Variants. Previous mutagenesis studies on the roles of Lys136 and Lys139 of antithrombin in heparin binding (*14, 15*) were done with variants having the wild-type sequence (Asn135/Ser137) of the Asn135 glycosylation site. In vivo and also in most expression systems in vitro, this sequence leads to the synthesis of a mixture of α- and β-forms of the inhibitor with different basal affinities for heparin. The α-form, being fully glycosylated, has a lower heparin affinity than the β-form, lacking carbohydrate on Asn135 (*21*−*24, 31*). To eliminate the influence of this glycoform heterogeneity from our studies, we expressed the Lys136 and Lys139 mutations on a pure β (N135A) background and the Lys139 mutation also on a pure α (S137T) background by introducing secondary mutations at positions 135 or 137.

The five variants, N135A, K136A/N135A, K139A/N135A, S137T, and K139Q/S137T, were more than 95% homogeneous in SDS−PAGE and nondenaturing electrophoresis (not shown). The N135A variant had a slightly higher mobility than the S137T variant in SDS−PAGE under both reducing and nonreducing conditions, consistent with the absence of one oligosaccharide chain and in agreement with previous work (*20*). The K136A/N135A and K139A/N135A variants migrated similarly to the N135A control and the K139Q/S137T variant similarly to the S137T control in SDS−PAGE under both reducing and nonreducing conditions. However, the three Lys variants had a slightly higher mobility than their respective control variants under nondenaturing conditions at alkaline pH, consistent with the loss of positive charge. The K136A/N135A variant eluted from immobilized heparin at 2.1 M NaCl, somewhat before the N135A variant at 2.5 M NaCl. In contrast, no difference in elution between the K139Q/S137T and S137T variants could be detected, both appearing at around 1.8 M NaCl.

Stoichiometric titrations, monitored by tryptophan fluorescence, of the N135A, K136A/N135A, S137T, and K139Q/S137T antithrombin variants with full-length heparin at $I = 0.15$, pH 7.4, and high antithrombin concentrations gave heparin to antithrombin binding stoichiometries in the range from 0.66 to 0.82 for the four variants. All these variants showed the normal ∼35% heparin-induced enhancement of intrinsic fluorescence in the titrations. Correspondingly, thrombin to antithrombin binding stoichiometries of 0.54−0.64 were obtained for these variants by titrations monitored by the loss of thrombin activity in the absence of heparin. In contrast with the analyses of these four variants, titrations of the K139A/N135A variant gave a heparin binding stoichiometry of only 0.26 and a fluorescence enhancement of only about 15%. As in previous work with other recombinant antithrombin variants, all preparations thus contained some inactive, probably latent, inhibitor (*18, 20*). Latent antithrombin, in which the intact reactive-bond loop is inserted into the A sheet, is inactive and binds heparin very weakly (*32, 33*). The heparin affinity is presumably comparable with that of reactive-bond-cleaved antithrombin, in which the loop is similarly inserted, i.e., ≥1000-fold lower than the affinity of the intact inhibitor (*34*). The inactive material is a minor component of the preparations of the N135A, K136A/N135A, S137T, and K139Q/S137T variants, but is dominating in the K139A/N135A preparation. The large amount of inactive protein in the latter preparation also accounts for the low heparin-induced fluorescence enhancement. For each variant preparation, the active antithrombin concentrations used in the studies of heparin and proteinase binding presented below were those obtained by the stoichiometric heparin and thrombin titrations, respectively.

Heparin Binding Affinity. Dissociation equilibrium constants, K_d, for the binding of full-length heparin and pentasaccharide to the antithrombin variants were determined at pH 7.4 and ionic strengths 0.3 or 0.4 by fluorescence titrations at low protein concentrations (Table 1). Most interactions at physiological ionic strength were too tight to be accurately quantified. As amply documented in previous work with plasma and recombinant antithrombins (*4, 18, 20*), full-length heparin bound about 5-fold more tightly than the pentasaccharide to both the N135A and S137T control

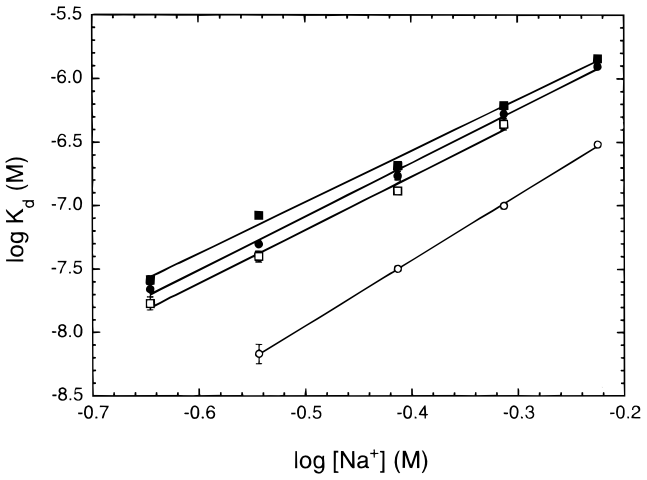

FIGURE 1: Sodium ion concentration dependence of dissociation equilibrium constants for full-length heparin and pentasaccharide binding to the N135A and K136A/N135A antithrombin variants at 25 °C, pH 7.4. (○) Full-length heparin, N135A; (□) pentasaccharide, N135A; (●) full-length heparin, K136A/N135A; and (■) pentasaccharide, K136A/N135A. Average values ± SE of at least three determinations are shown. Error bars not shown lie within the dimensions of the symbols. The solid lines represent linear regression fits.

variants. The K136A mutation resulted in an appreciable (5−7-fold) reduction in affinity for full-length heparin, compared with the N135A control antithrombin, an affinity difference in agreement with the different elution positions of the two variants in heparin affinity chromatography. However, the decrease in pentasaccharide affinity was much smaller (1.5−2-fold), and the full-length heparin and pentasaccharide affinities of the K136A/N135A variant were therefore nearly equal. In contrast, the affinity measured for the binding of full-length heparin to the K139A/N135A mutant was indistinguishable from that for the N135A control. Moreover, the affinity of the K139Q/S137T variant for both full-length heparin and pentasaccharide was minimally (1.2−1.3-fold) decreased, compared with the S137T control, consistent with both variants eluting similarly from immobilized heparin. Because of these negligible effects, heparin binding to the K139A/N135A and K139Q/S137T variants was not characterized in further detail.

The ionic and nonionic contributions to the binding of full-length heparin and pentasaccharide to the N135A and K136A/N135A antithrombin variants at pH 7.4 were evaluated from the dependence of the observed dissociation equilibrium constants on sodium ion concentration, as described previously (*4, 18, 20, 35*). Plots of log K_d vs log [Na^+] were linear for the binding of both saccharides to the two variants (Figure 1). The number of ionic interactions involved in the binding and the nonionic contribution to the binding were obtained from the slopes and intercepts, respectively, of these plots (Table 2). About six charge interactions participated in full-length heparin binding to the N135A variant, in agreement with previous studies (*18, 20*), whereas only five such interactions were involved in the binding to the K136A/N135A variant. In contrast, about five ionic interactions were found to be involved in the binding of the pentasaccharide both to the control N135A variant, as in earlier work (*18, 20*), and to the K136A/N135A variant. The K136A mutation thus resulted in a loss of one ionic interaction with full-length heparin but in no change of ionic interactions with the pentasaccharide. The nonionic contributions to the binding of full-length heparin and pentasaccharide to the control and K136A/N135A variants were similar.

Table 2: Ionic and Nonionic Contributions to Full-Length Heparin and Pentasaccharide Binding to the N135A and K136A/N135A Antithrombin Variants at 25 °C, pH 7.4[a]

heparin form	antithrombin variant	Z	log K_d'
H26	N135A	6.4 ± 0.1	−5.4 ± 0.1
	K136A/N135A	5.3 ± 0.2	−5.0 ± 0.1
H5	N135A	5.3 ± 0.3	−5.1 ± 0.1
	K136A/N135A	5.0 ± 0.2	−4.9 ± 0.1

[a] The number of ionic interactions (Z) involved in the binding of heparin to the antithrombin variants and the nonionic contribution (log K_d') to the binding were determined from the slopes and intercepts, respectively, of the plots in Figure 1. Errors represent ± SE obtained by linear regression.

Kinetics of Heparin Binding. The kinetics of binding of full-length heparin and pentasaccharide to the N135A control and K136A/N135A antithrombin variants were investigated at pH 7.4 and ionic strengths of 0.3 and 0.4 by stopped-flow fluorescence under pseudo-first-order conditions at low heparin concentrations. Under these conditions, the pseudo-first-order rate constant, k_{obs}, increases linearly with heparin concentration, the slope of this plot giving the overall association rate constant, k_{on}, and the intercept on the ordinate the overall dissociation rate constant, k_{off} (*4, 18, 20, 36*). The k_{off} values at ionic strength 0.4 are more accurate and thus more reliably reveal possible differences, as k_{off} increases and k_{on} decreases with ionic strength, leading to a smaller error in the intercept (*4, 20*). As shown previously for plasma and recombinant antithrombin (*4, 20*), both k_{on} and k_{off} were lower for full-length heparin than for pentasaccharide binding to the two variants (Table 1). The K136A mutation resulted in a ∼2-fold lower k_{on} and a ∼2-fold higher k_{off} for the binding of full-length heparin, in agreement with the observed ∼5-fold decrease in affinity. A minor, about 1.2-fold, decrease in k_{on} was seen for pentasaccharide binding, partly accounting for the small measured decrease in affinity, whereas no clear difference in k_{off} was apparent. Dissociation equilibrium constants were calculated as k_{off}/k_{on}, and these calculated values agreed well with those measured by the fluorescence titrations (Table 1).

Kinetics of Proteinase Inhibition. Second-order rate constants for thrombin and factor Xa inhibition by the N135A, K136A/N135A, S137T, and K139Q/S137T antithrombin variants and by the complexes of these variants with full-length heparin or pentasaccharide were determined at pH 7.4 and ionic strength 0.15 by discontinuous assays of residual proteinase activity. All rate constants for thrombin inhibition measured without heparin were unaffected by Polybrene (*18, 20*), excluding a possible heparin contamination of the antithrombin preparations. The rate constant for inhibition of thrombin in the absence of heparin was similar for the N135A and S137T variants, whereas this rate constant for factor Xa inhibition was about 2-fold higher for the N135A variant than for the S137T variant, as shown previously (*20, 21*) (Table 3). The rate constants for proteinase inhibition both by antithrombin alone and by the complexes of the inhibitor with full-length heparin or pentasaccharide were essentially unaffected by the K136A or K139Q mutations (Table 3). The pentasaccharide en-

Table 3: Association Rate Constants for Proteinase Inhibition by the N135A, K136A/N135A, S137T, and K139Q/S137T Antithrombin Variants Alone and in Complex with Full-Length Heparin or Pentasaccharide at 25 °C, pH 7.4, and Ionic Strength 0.15[a]

proteinase	antithrombin variant	$10^{-3} \times k_{uncat}$ ($M^{-1}\cdot s^{-1}$)	$10^{-6} \times k_{H26}$ ($M^{-1}\cdot s^{-1}$)	$10^{-5} \times k_{H5}$ ($M^{-1}\cdot s^{-1}$)
thrombin	N135A	9.4 ± 0.4	9.0 ± 0.5	nd[b]
	K136A/N135A	10.7 ± 0.3	8.9 ± 0.7	nd
	S137T	8.4 ± 0.1	8.2 ± 0.3	nd
	K139Q/S137T	11.1 ± 0.1	8.3 ± 0.4	nd
factor Xa	N135A	4.8 ± 0.2	1.2 ± 0.04	6.1 ± 0.2
	K136A/N135A	3.8 ± 0.1	0.94 ± 0.03	5.4 ± 0.2
	S137T	2.3 ± 0.1	0.64 ± 0.01	3.8 ± 0.2
	K139Q/S137T	2.7 ± 0.1	0.90 ± 0.03	4.1 ± 0.1

[a] Second-order association rate constants for reactions of the uncomplexed antithrombin variants (k_{uncat}) and the complexes of the variants with full-length heparin (k_{H26}) or pentasaccharide (k_{H5}) with proteinases were determined as described in (*18*). The k_{uncat} values are averages ± SE of at least three determinations. Values of k_{H26} and k_{H5} ± SE were obtained by linear regression of plots of k_{obs} vs heparin concentration, comprising 4−6 points in the 0.1−8 nM concentration range. [b] nd, not determined.

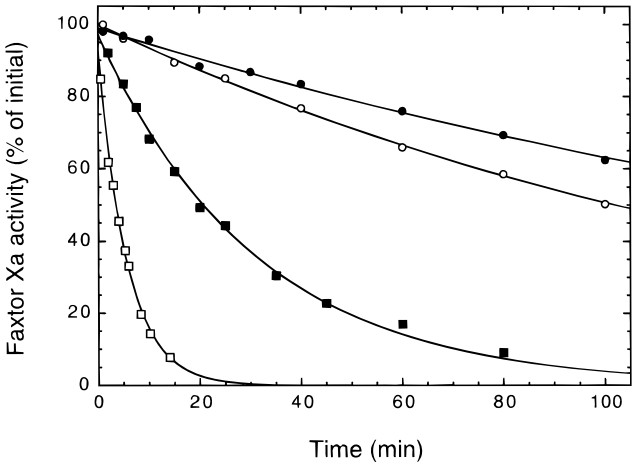

FIGURE 2: Rate of inhibition of factor Xa by the N135A and K136/N135A antithrombin variants in the absence and presence of full-length heparin at 25 °C, pH 7.4, and ionic strength 0.4. (○) N135A alone; (□) N135A with heparin; (●) K136A/N135A alone; and (■) K136A/N135A with heparin. The antithrombin and heparin concentrations were 10 nM and the factor Xa concentration 1 nM. The solid lines represent linear regression fits to a single-exponential decay function with an endpoint of zero activity.

hancement of the rate of thrombin inhibition is minimal, less than 2-fold (*4*, *20*), and therefore was not investigated.

The functional consequence of the contribution of Lys136 to the affinity of antithrombin for full-length heparin was illustrated by analyses of the kinetics of factor Xa inhibition by the K136A/N135A and N135A variants in the presence of the polysaccharide at ionic strength 0.4. At this ionic strength, the heparin affinities of both variants are sufficiently low that the concentrations of their complexes with heparin at the concentrations necessary for reasonable experimental accuracy are governed by these affinities. Due to the higher heparin affinity of the N135A variant, resulting in a higher concentration of the complex with the polysaccharide, this variant inhibited factor Xa considerably faster than the K136A/N135A variant (Figure 2). The pseudo-first-order rate constants for the heparin-catalyzed reaction under the conditions used, corrected for the contribution of the uncatalyzed factor Xa inhibition (Figure 2), were 2.9×10^{-3} and 4.7×10^{-4} s^{-1} for the N135A and K136A/N135A variants, respectively. These values, reflecting about a 6-fold difference in heparin-catalyzed inactivation rate, are in good agreement with those expected from the heparin affinities (*4*, *20*).

DISCUSSION

In this work, we have investigated the roles of lysines 136 and 139 of antithrombin in heparin binding. These two residues have been suggested to be part of an extended heparin binding site adjacent to the specific pentasaccharide binding region (*14*, *15*, *17*). This site most likely binds saccharides outside the pentasaccharide sequence in full-length heparin chains, thereby being responsible for the higher affinity of antithrombin for such chains. However, an interaction with the extended binding site apparently is not required for the heparin-induced allosteric activation of the inhibitor necessary for the increased rate of factor Xa inhibition, nor for the bridging effect involved in acceleration of thrombin inhibition (*4*, *19*, *20*).

A study in which lysine residues of antithrombin were chemically modified initially suggested that Lys136 is important for heparin binding (*11*). Moreover, the crystal structure of antithrombin in complex with a heparin pentasaccharide is consistent with Lys136 participating in binding of long heparin chains (*17*). However, a study of a recombinant antithrombin variant with a Lys136 to Thr substitution concluded that this replacement did not affect heparin affinity, although heparin acceleration of thrombin inhibition by the variant was slightly impaired (*14*). In an attempt to resolve this apparent controversy, we have characterized the affinity and kinetics of full-length heparin and pentasacharide binding to a recombinant antithrombin variant with Lys136 replaced by Ala on the N135A β background.

Our results show that Lys136 of antithrombin participates in binding of full-length heparin chains but is not critical for pentasaccharide binding. The Lys136 to Ala substitution thus led to an appreciable decrease in the affinity of the recombinant antithrombin for full-length heparin, corresponding to a decrease in binding energy of about 5 $kJ\cdot mol^{-1}$. In contrast, only a minor reduction in pentasaccharide affinity was observed, and only a small part of this decrease could be verified by kinetic analyses. The loss in affinity for full-length heparin is of such a magnitude that it probably would have been difficult to detect by the solid-phase assay used in the previous work (*14*). The glycosylation heterogeneity and associated heterogeneity in heparin affinity resulting from the use of the wild-type Asn135−Ser137 sequence in the previous studies may also have obscured the effect of a mutation at Lys136. As a result of the different effects on full-length heparin and pentasaccharide binding, the affinities of the mutant for the two saccharides were nearly comparable. Moreover, the reduced affinity caused by the Lys136 to Ala mutation was due to the loss of one charge interaction with full-length heparin, whereas the number of ionic interactions with the pentasaccharide and the nonionic contributions to either interaction were unaltered. In addition, the mutation did not influence the rates of inhibition of thrombin and factor Xa by either uncomplexed antithrombin

or the complexes of the inhibitor with pentasaccharide or full-length heparin, suggesting that it did not affect the native antithrombin conformation and also that Lys136 is not required for the heparin-induced allosteric activation of the inhibitor or for the bridging effect. Together, these data indicate that Lys136 of antithrombin is located in the extended binding site outside the pentasaccharide binding region that accounts for the increased affinity of the inhibitor for long heparin chains. In contrast, Lys136 can at most exert a very weak, long-range effect on pentasaccharide binding. As demonstrated by kinetic analyses, the contribution of Lys136 to the affinity of antithrombin for full-length heparin is of appreciable functional importance for heparin acceleration of proteinase inhibition under conditions at which the concentration of the heparin-antithrombin complex is critically dependent on this affinity.

In previous studies, mutation of either Arg132 or Lys133 of antithrombin was shown to have similar consequences for heparin affinity as the Lys136 to Ala substitution in this work (*19*). Each of the two former mutations thus resulted in a substantial decrease in affinity for full-length heparin but in a much smaller reduction in pentasaccharide affinity. Accordingly, the affinity of each mutant for full-length heparin was only somewhat higher than that for the pentasaccharide. Each mutation also led to the loss of one ionic interaction with full-length heparin, thereby reducing the number of such interactions to that observed for the pentasaccharide. These findings are consistent with Arg132, Lys133, and Lys136 all being located in the extended heparin binding site of antithrombin, as previously concluded for Arg132 and Lys133 (*19, 32*). In this site, the three positively charged residues appear to act cooperatively, together providing one effective charge interaction with a negatively charged group in a saccharide unit close to the pentasaccharide sequence in long heparin chains, or possibly with several cooperatively acting such groups. Because of the cooperativity, the absence of only one of the three positively charged side chains is sufficient to abolish all or most of the binding energy contributed by this interaction. Moreover, removal of all three charged side chains would not be expected to decrease the binding energy to a greater extent than removal of only one of them. The location of Arg132, Lys133, and Lys136 in the extended heparin binding site and their apparent cooperativity are supported by the crystal structure of the antithrombin−pentasaccharide complex (*17*). This structure shows that all three residues are located in the one and one-half turn extension of the D-helix induced by pentasaccharide binding. They form a positively charged surface on the outward-facing side of this extension that is appropriately positioned to interact with a heparin chain just beyond the pentasaccharide region.

A kinetic characterization showed that the decrease in affinity for full-length heparin caused by the Lys136 to Ala mutation was due to both a lower bimolecular association rate constant, k_{on}, and a higher dissociation rate constant, k_{off}. The decrease in k_{on} was sufficiently small to preclude an assessment whether it was caused by a higher dissociation equilibrium constant of the initial heparin binding step or by a lower forward rate constant of the conformational change step (*4, 18, 20*). However, the increased k_{off} shows that, when long heparin chains bind to antithrombin, Lys136 participates in maintaining the inhibitor in the conformationally altered state by decreasing the rate of reversal of the conformational change (*4, 18, 20*), although this contribution is also small. This effect is presumably due to the helix D extension positioning Lys136, and the other residues in the extended binding site, for optimal interaction with full-length heparin, as discussed above.

Lys139 has been implicated in binding of long heparin chains by studies of a recombinant variant in which this residue was changed to Gln (*14, 15*). This substitution was reported to result in a large decrease in affinity for heparin as well as in a pronounced defect in the heparin-accelerated rate of thrombin inhibition (*14*) but to have no effect on pentasaccharide acceleration of factor Xa inhibition (*15*). These results are surprising given that Lys139 is located in strand 2A at the edge of the A β-sheet, at an appreciable distance (~20 Å) from the Arg132−Lys133−Lys136 cluster on helix D, in the crystal structure of the antithrombin−pentasaccharide complex (*17*). Because of this location, it is unlikely to be involved even in the interaction with full-length heparin chains.

We found that substitution of Lys139 by Ala on the N135A β background did not detectably affect the affinity of antithrombin for full-length heparin. However, this variant had a low heparin binding stoichiometry and a low fluorescence increase on heparin binding, indicative of an appreciable amount of inactive, latent antithrombin being formed due to a decreased stability of the native protein. We therefore also characterized a more stable variant, in which Lys139 was replaced by Gln on the S137T α background. A similar α isoform presumably was the main species in the previously studied Lys139 to Gln mutant, which was expressed with the wild-type sequence of the Asn135 glycosylation site (*14*). In contrast to the previous work, we found that the Lys139 to Gln replacement caused only a negligible about 1.2-fold reduction in antithrombin affinity for both pentasaccharide and full-length heparin. The different results of the two studies may be due to the fact that the dissociation constants for heparin binding in the previous work were measured by a nonequilibrium, solid-phase assay, in which the coating of the mutant on the walls of plastic wells may have adversely affected heparin affinity. Our binding data thus show that Lys139 is not involved in antithrombin binding of either pentasaccharide or full-length heparin, in agreement with its location away from both the pentasaccharide and the extended heparin binding sites, as discussed previously. The similar rate constants for inhibition of thrombin and factor Xa by the Lys139 to Gln mutant and the control in complex with pentasaccharide or full-length heparin also indicate that Lys139 is not involved in the heparin-induced conformational change of antithrombin and in the bridging effect.

The substitution of K136 was done on the N135A β-antithrombin background, in which the carbohydrate side chain on Asn135 is lacking, whereas substitution of K139 was done both on the β background and on the fully glycosylated S137T α background. Several lines of evidence indicate that the use of the two differently glycosylated antithrombin forms has not influenced the results. Such an influence would have been possible if the N135 carbohydrate side chain had affected the interaction of full-length heparin with the extended binding site. Most importantly, however, identical results were obtained for substitution of K139 on

both backgrounds. Moreover, previous observations have shown that the oligosaccharide side chain on Asn135 decreases the affinities of antithrombin for full-length heparin and pentasaccharide to the same extent, by affecting the forward and reverse rate constants of the conformational change induced in the second binding step (*20*). Such similar effects of the two saccharides on the affinity, arising only from effects on the conformational change, are inconsistent with the carbohydrate side chain interfering with the binding of full-length heparin in an extended binding site. In addition, the oligosaccharide on Asn135 has been shown not to influence the number of charges participating in the interaction with either pentasaccharide or full-length heparin (*20*), which also would not be the case if the oligosaccharide side chain affects the ionic interaction with full-length heparin chains in an extended site. Therefore, our results strongly indicate that Lys136 participates in the binding of full-length heparin chains and Lys139 does not interact with such chains, regardless of the presence of a carbohydrate side chain on Asn135.

In conclusion, the results of this work further define the extended heparin binding site of antithrombin, showing that Lys136 is located in this site whereas Lys139 is not. They also suggest that Lys136 acts in concert with Arg132 and Lys133 to provide one effective charge interaction with one or more negatively charged groups in saccharide units outside the pentasaccharide sequence of long heparin chains. Thereby, Lys136 contributes to increasing the affinity of antithrombin for such heparin species and thus to increasing the heparin-accelerated rate of proteinase inhibition at low antithrombin and heparin concentrations. However, Lys136 does not have a critical role in the conformational change or the bridging effect that are involved in heparin acceleration of proteinase inhibition.

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