Role of Arginine 132 and Lysine 133 in Heparin Binding to and Activation of Antithrombin*

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The binding of heparin to antithrombin greatly accelerates the rate of inhibition of the target proteinases thrombin and factor Xa. Acceleration of the rate of inhibition of factor Xa involves a conformational change in antithrombin that is translated from the heparin binding site to the reactive center loop. A mechanism has been proposed for generation and propagation of the conformational change in which the binding of the negatively charged heparin reduces ionic repulsions between positively charged residues on and adjacent to the D-helix in the heparin binding site of antithrombin (van Boeckel, C. A. A., Grootenhuis, P. D. J., and Visser, A. (1994) Nature Struct. Biol. 1, 423–425). This charge neutralization is proposed to elongate the D-helix and initiate the conformational change which is then translated to the reactive center loop. Several basic residues, including arginine 132 and lysine 133, were predicted to be important both in heparin binding and in this mechanism of heparin activation. To test both the helix extension mechanism and the role of these two residues in heparin binding and factor Xa inhibition, we individually mutated arginine 132 and lysine 133 to uncharged methionine by site-directed mutagenesis. The $K_d$ values for binding of R132M and K133M variants to the high affinity pentasaccharide were weakened only 2.3- and 4.5-fold respectively, suggesting a location for R132 and K133 peripheral to the main pentasaccharide binding site. However, the $K_d$ values for long chain high affinity heparin were weakened at least 17-fold for both R132M and K133M, indicating involvement of each residue in binding extended chain heparin species. These reductions in affinity were ionic strength-dependent. The rates of inhibition of factor Xa and thrombin by each variant, however, were indistinguishable from those of control antithrombin, and the accelerations of the rate of inhibition produced by heparin were normal. We conclude that neither arginine 132 nor lysine 133 plays an important role in the binding of heparin pentasaccharide or in the mechanism of heparin activation, suggesting that D-helix extension through charge neutralization is not the mechanism for transmission of conformational change from the heparin binding site to the reactive center region. Arginine 132 and lysine 133 do, however, play a role in tight binding of longer chain heparin species through ionic interactions.

Antithrombin is a member of the serpin superfamily and is the principal inhibitor of thrombin and factor Xa, serine proteinases involved in the blood coagulation cascade. Antithrombin forms a 1:1 covalent complex with the proteinase through the reactive center loop on antithrombin. Heparin, a highly negatively charged glycosaminoglycan, functions as an anticoagulant by binding antithrombin and greatly accelerates the rate of inhibition of target proteinases (for review, see Ref. 1). There are two mechanisms of heparin acceleration of inhibition that depend on the proteinase. In the case of thrombin, heparin performs a bridging function, binding to both antithrombin and thrombin (2). Activation therefore requires a long chain heparin that also contains the high affinity pentasaccharide sequence for optimal increase in the rate of inhibition of thrombin. For factor Xa, the acceleration results almost completely from a heparin-induced conformational change in antithrombin (3). A specific pentasaccharide sequence in heparin binds to antithrombin, causing the antithrombin to undergo a conformational change that is translated from the heparin binding site to the reactive center loop (4). This conformational change enhances reactivity toward factor Xa by making the reactive center loop on antithrombin more accessible for reaction with factor Xa (3).

The heparin binding site on antithrombin has been approximately localized, based on properties of variants and chemical modification studies, to a region of positively charged residues located on the D-helix and in the immediately adjacent amino-terminal region (5, 6). These residues include several arginines (arginine 24 (7), arginine 47 (8), and arginine 129 (9)) and several lysines (lysine 107, lysine 125, and lysine 136 (10–12)). Recently van Boeckel and colleagues have proposed a mechanism for heparin activation of antithrombin in which the binding of heparin stabilizes and elongates the D-helix in the heparin binding site by reducing ionic repulsion between the positively charged lysine and arginine residues located on the same face of the D-helix and on the adjacent, less structured stretch of polypeptide. The elongation of the D-helix translates the conformational change to the reactive center as a result of the newly extended helix being attached to a strand of $\beta$-sheet A, resulting in expulsion of the P15 and P14 residues of the reactive center loop from $\beta$-sheet A (13) (Fig. 1). From molecular modeling, these authors specifically implicated eight basic residues as critical for heparin pentasaccharide binding and transmission of the conformational change, including arginine 132 and lysine 133. Although there is evidence from chemical modification studies or from the properties of antithrombin variants to implicate most of the proposed basic residues, no direct evidence has been put forward to implicate arginine 132

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The abbreviations used are: serpin, serine protease inhibitor; K125M, R132M, and K133M, antithrombin variants in which lysine at position 125, arginine at position 132, or lysine at position 133, respectively, has been replaced by methionine.

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alter the conformation at the P1–P1 interface, thereby promoting expulsion of residues P15 and P14, which in turn neutralizes the helix to extend, causing tension on strand s2A, and between the basic residues. Upon heparin binding the charges are neutralized to the end of the D-helix is disordered due to charge repulsion.

The resulting triplicates correspond to the arginine 132 to methionine and lysine 133 to threonine in what has been proposed to be an extended heparin binding site (14).

To investigate the role of arginine 132 and lysine 133 in binding to pentasaccharide and longer chain heparin species and in the mechanism of activation by heparin, these residues were individually mutated to methionine and the resulting antithrombin variants characterized. Mutation to methionine was chosen to give an uncharged but approximately isosteric replacement for both arginine and lysine. We found that mutation of either of these residues resulted in only a small reduction in affinity of antithrombin for heparin pentasaccharide, indicating that neither residue is essential for pentasaccharide binding. The affinity of longer chain heparin was reduced at least 17-fold for both R132M and K133M in an ionic strength-dependent manner. This suggests a role for these residues in binding longer chain heparin species through additional ionic interactions. Normal rates were seen for inhibition of factor Xa, both in the absence and presence of pentasaccharide or full-length high affinity heparin, suggesting that these residues are not involved in the mechanism of activation of antithrombin and therefore that D-helix extension through charge neutralization is unlikely to be the means of transmission of conformational change from the heparin binding site to the reactive center. The rate of inhibition of thrombin in the presence of full-length heparin was also normal, implying also that the bridging mechanism does not depend on either of these charged residues.

MATERIALS AND METHODS

Production and Isolation of Variant Forms of Antithrombin—Site-directed mutagenesis of human antithrombin cDNA on an N135Q background was carried out in M13mp19 as described previously (4), using a single stranded uracil-containing template and the antisense oligonucleotide 5'-TTT GTG CTT CAT ATA GAG TCG GCA-3' and 5'-GGA TTT GTC CAT GAG AAG TCG-3' (the underlined triplets correspond to the arginine 132 to methionine and lysine 133 to methionine changes, respectively). The N135Q background results in antithrombin that lacks carbohydrate at position 135. The resulting antithrombin thus resembles the β-form that occurs naturally in plasma and that has a higher affinity for heparin (15–17). For antithrombin variants in which mutations are introduced that greatly reduce heparin affinity, this has the advantage that the heparin affinity is still high enough to be readily measurable. The mutated antithrombin cDNAs were ligated into pMASHop as described (4) to generate plasmids pMAAT3-K133M-N135Q, R132Q, and pMAAT3-K133M-N135Q. Rh hamster kidney cells were stably transfected with pRMH140 and pSV2dhfr and either pMAAT3-R132M-N135Q or pMAAT3-K133M-N135Q. Transfected cells were selected by resistance to neomycin (Life Technologies, Inc.) and methotrexate (Sigma) as described previously (18). Antithrombin was isolated from the serum-free growth medium of stably transfected cells by affinity chromatography on a heparin-Sepharose column and size exclusion chromatography on Sephacryl S-150. The N135Q mutation results in two antithrombin glycoforms with 2-fold difference in heparin affinity (17). These have been designated H and L forms. The glycoform of R132M and K133M used in the present studies was the L form. Comparisons are therefore reported relative to a control of N135Q L glycoform recombinant antithrombin.

Antithrombin Assay—The level of antithrombin present during the production and purification procedures was monitored by radial immunodiffusion assay, using radial immunodiffusion assay plates containing sheep anti-human antithrombin antibody (The Binding Site Ltd., Birmingham, UK).

SDS-Polyacrylamide Gel Electrophoresis—The ability of the mutant antithrombins to form a covalent complex with proteinase was determined by electrophoresis on a 10% SDS-polyacrylamide gel. Prior to electrophoresis, the antithrombin and thrombin were incubated for 30 min on ice in the presence of heparin as a catalyst. Phenylmethylsulfonyl fluoride was added to inactivate any unreacted thrombin, and the samples were incubated for 5 min on ice. Sample buffer containing dithiothreitol was added, and the samples were boiled for 5 min. Protein bands were visualized with Coomassie Brilliant Blue.

Stoichiometry of Inhibition—The stoichiometry of inhibition in the absence of heparin was measured by incubating different concentrations of antithrombin (10–100 nM) with 100 nM factor Xa. The reactions were carried out in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000, and 0.1 M NaCl (I 0.15) at 25 °C. Reactions were allowed to go to completion and residual factor Xa activity was measured by diluting the reaction mixture 100-fold with 100 μM Spectrozyme Xa (American Diagnostica, Greenwich, CT) and measuring the rate of substrate hydrolysis at 405 nm as described previously (19). The stoichiometry of inhibition was determined by plotting the residual proteinase activity against the ratio of antithrombin to factor Xa. Factor Xa activity was determined by titration with plasma antithrombin of known activity.

Determination of Heparin-Antithrombin Dissociation Constants—Heparin binding studies were carried out as described previously (20) for both full-length high affinity heparin and high affinity heparin pentasaccharide. Heparin was titrated into antithrombin, and the binding was monitored by the change in endogenous tryptophan fluorescence, measured on an SLM 8000 spectrophotometer at 280 nm, and observing emission at 340 nm. The excitation width was 4 nm and the emission bandwidth was 16 nm. For determination of the full-length high affinity heparin-variant antithrombin dissociation constants, 50 nM antithrombin was used at I 0.1, 0.125, 0.15, 0.2, and 0.25 and 500 nM at I 0.3, for pentasaccharide-antithrombin dissociation constants, 300 nM antithrombin was used. For the control N135Q titration with high affinity full-length heparin, the binding was too tight at I 0.15 to determine an accurate value for the dissociation constant. To obtain an estimate of the value at I 0.15, dissociation constants were measured using 50 nM antithrombin at I 0.2, 0.25, 0.3, and 0.35 and 100 nM antithrombin at I 0.4 and the values extrapolated to I 0.15. For the control N135Q antithrombin titration with proteinase antithrombin at I 0.15, 50 nM antithrombin was used. Titrations were carried out at 25 °C in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000, and appropriate NaCl concentrations to give the desired final ionic strength. Data were fitted to a single binding model equation by nonlinear least squares analysis using the SCIENTIST program (Micromath Scientific Software, Salt Lake City, UT).

Ionic Strength Dependence of Heparin-Antithrombin Dissociation Constants—Analysis of the role of ionic interactions in heparin binding to the two variant antithrombins used the polyelectrolyte theory of Manning and Record (21). For a linear polyelectrolyte (heparin) binding to a Z-valent positively charged binding site on a protein (antithrombin), the relationship between the observed Kd (Kd,obs) for the protein-polyelectrolyte complex and the concentration of displaced monovalent counterion (Na+) is given by Equation 1:

\[
\log K_{d,obs} = \log K_d + Z \varphi \log [Na^+] 
\]

(1)
Heparin Binding Site of Antithrombin

![Diagram](Image)

**Fig. 2.** Homogeneity of recombinant antithrombin variants and demonstration of their ability to form stable covalent complexes with thrombin. 10% SDS-polyacrylamide gel electrophoresis of antithrombin and antithrombin-thrombin reactions. Lane 1, thrombin alone. – and +, antithrombin in the absence and presence, respectively, of thrombin. The antithrombin used is indicated above each pair of lanes. Some unreacted antithrombin is visible in lanes 3, 5, and 7; however, stoichiometry titrations indicate that each antithrombin is fully active, with a stoichiometry of inhibition of 1.

and Ψ is the fraction of counterion bound per charged site on the polyelectrolyte and that is released upon formation of complex with the protein. For heparin this has been estimated to be ~0.8 (22).

**Kinetic Assays—** The rate of inhibition of factor Xa by antithrombin was measured under pseudo-first order conditions of 1 μM antithrombin and 10 nM factor Xa. The pseudo-first order rate constant \(k_{\text{obs}}\) was obtained from a semilog plot of residual factor Xa activity against time of incubation. Residual factor Xa activity was determined by diluting the antithrombin-factor Xa reaction mixture 10-fold with 100 μl Spectrozyme Xa (American Diagnostica) and measuring the rate of substrate hydrolysis at 405 nm as described previously (19). The second-order rate constant in the absence of heparin \(k_{\text{cat}}\) was obtained by dividing \(k_{\text{obs}}\) by the concentration of antithrombin. For the heparin catalyzed reaction, the second order rate constant \(k_{\text{cat}}/K_d\) was determined using 250 nM antithrombin and 5 nM factor Xa in the presence of catalytic concentrations of heparin using Equation 2 (20):

\[
k_{\text{obs}} = \frac{k_{\text{cat}} \cdot [H]}{K_h} + \frac{[AT]}{K_{AT} + [AT]},
\]

where \([H]\) is the total heparin concentration, \([AT]\) is the total antithrombin concentration, \(K_h\) is the measured antithrombin-heparin dissociation constant, and \(K_{AT}\) is the second-order rate constant for inhibition of proteinase in the absence of heparin. The heparin catalyzed reactions were carried out in polyethylene glycol-coated cuvettes.

Rates of inhibition of thrombin by antithrombin in the absence and presence of heparin were determined similarly using 1 μM antithrombin and 50 nM thrombin in the absence of heparin and 250 nM antithrombin and 5 nM thrombin in the presence of high affinity heparin. Residual thrombin activity was measured by the rate of hydrolysis of the chromogenic substrate, S2238 (Chromogenix) monitored spectrophotometrically at 405 nm. All kinetic reactions were carried out at 25 °C in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1% polyethylene glycol 8000, and 0.1 μM NaCl (I 0.15).

**Materials—** Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Life Technologies, Inc. Human factor Xa was a gift from Dr. Paul Bock, Vanderbilt University. Thrombin was prepared from outdated human plasma by the method of Milewich et al. (23). Full-length high affinity heparin, \(H_0\), was a gift from Dr. Steven T. Olson, University of Illinois-Chicago. Synthetic high affinity heparin pentasaccharide was a gift from the late Dr. Jean Choay, Centre Choay, The plasmids pMASstop, pRMH140, and pSV2dhfr (18) were gifts from Dr. Gerd Zettlemeissl, Behringwerke, Marburg.

**RESULTS**

**Integrity of R132M and K133M Antithrombin—** R132M and K133M antithrombins were shown to be homogenous and able to form stable covalent complex with proteinase by analysis on an SDS-polyacrylamide gel (Fig. 2). These antithrombin variants migrated with the same mobility as the control N135Q antithrombin in the absence of proteinase. When the mutants were preincubated with an excess of thrombin in the presence of heparin, both R132M and K133M were able to form a stable antithrombin-thrombin complex, as seen from the higher molecular weight band on the gel (Fig. 2). The formation of a stable covalent complex with proteinase indicated that the heparin binding site mutations had not adversely affected the serpin inhibition mechanism. We also measured the stoichiometry of inhibition, defined as the number of moles of inhibitor required to inhibit 1 mol of proteinase, for inhibition of factor Xa in the absence of heparin. No difference was seen between control and variant antithrombins, all of which gave a 1:1 stoichiometry of inhibition (data not shown).

**Heparin Binding to R132M and K133M Antithrombin Variants—** Titration of full-length heparin into each variant antithrombin at high antithrombin concentration, 0.5 μM at I 0.15, gave the expected 1:1 stoichiometry of binding and resulted in normal enhancement of endogenous tryptophan fluorescence. Heparin dissociation constants were determined from titrations at lower antithrombin concentration, as described under ‘Materials and Methods.’ Each of the antithrombin mutations, R132→M and K133→M, resulted in a reduction in heparin affinity for both high affinity (full-length) heparin and heparin pentasaccharide compared to the control antithrombin (Table I and Fig. 3). However, the reduction in affinity at I 0.15 was very much smaller for heparin pentasaccharide (2.3–4.5-fold) than for full-length heparin (>17-fold), indicating that neither residue is critical for binding of the pentasaccharide. The reductions in affinity correspond to percentage changes in binding energy \(\Delta G\) of 5.0 and 8.7% for R132M and K133M binding to pentasaccharide and 14.5 and 14.3% for respective binding to full-length heparin.

To determine whether the larger reduction in affinity seen for binding of full-length heparin was due to loss of one or more ionic interactions, values of \(K_d\) for full-length heparin-antithrombin complexes were also determined at ionic strengths other than 0.15. A plot of log \(K_d\) against log I should be linear with a slope equal to \(Z \times \Psi\), where \(Z\) is the number of ionic interactions and \(\Psi\) is the screening constant, which for heparin has the value of 0.8 (22) (see under ‘Materials and Methods’). Both variants, as well as control antithrombin, gave linear plots, although with lower slope for the two variants (Fig. 4), indicating a reduction in the number of ionic interactions for binding of heparin by each of the two variants. A value for \(Z\) of 4.6 ± 0.5 was obtained for the control antithrombin, which compares favorably with the previously reported value of 4.8 ± 0.1 for plasma antithrombin (24). Values for \(Z\) of 3.4 ± 0.1 and 3.1 ± 0.2 were obtained for the R132M and K133M variants, respectively. The changes in ionic strength dependence corresponded to a loss of 1–1.5 ionic interactions for each of the variants, which within the errors of the determinations corresponds to ~1.

**Proteinase Inhibition by R132M and K133M Antithrombin in the Absence and Presence of Heparin—** The basal rates of inhibition of factor Xa and thrombin, as determined by discontinuous assay of residual proteinase activity, were unaffected by the R132M or K133M antithrombin mutations (Table II). Since the heparin accelerated rate of inhibition of factor Xa by antithrombin is dependent on a conformational change induced by heparin pentasaccharide binding, we also determined the rate of inhibition of factor Xa by complexes of the antithrombin variants with heparin to see if the mutations R132M and K133M had affected the ability of antithrombin to undergo the normal conformational change or the ability of heparin to fully activate antithrombin. The mutations had no effect on the ability of either heparin pentasaccharide or full-length heparin to fully activate antithrombin (Fig. 5 and Table II). We also measured the full-length heparin-catalyzed rate of inhibition of
thrombin to determine whether the loss of ionic interactions outside of the pentasaccharide binding site caused by mutation of R132 or K133 had affected the heparin bridging mechanism that is responsible for heparin acceleration of the inhibition of thrombin (3). No significant difference between control and variant antithrombins was found (Table II), indicating that the bridging mechanism is not affected.

**DISCUSSION**

In this work, we have examined whether arginine 132 and lysine 133 are directly involved in two aspects of heparin binding to antithrombin: first, whether either residue contributes critical ionic interactions to the binding of the high affinity pentasaccharide, and second, whether either residue is involved in a charge neutralization-helix extension mechanism of heparin activation of inhibition of factor Xa by antithrombin, as proposed by van Boeckel and colleagues (13). From the heparin binding properties of two recombinant variants, R132M and K133M, we found that neither arginine 132 nor lysine 133 appears to be critical for pentasaccharide binding. However, the ionic strength-dependent reductions in affinity for full-length heparin, larger than for heparin pentasaccharide for each of these antithrombin variants, suggests that arginine 132 and lysine 133 may be involved in additional ionic interactions outside of the pentasaccharide binding site. From the unchanged rates of inhibition of factor Xa by the two variants both in the absence and presence of heparin pentasaccharide, we conclude that neither arginine 132 nor lysine 133 is directly involved in the conformational change-dependent mechanism of heparin activation of antithrombin, thus suggesting that the proposed D-helix extension mechanism through charge neutralization may not be correct.

Arginine 132 and lysine 133 have been proposed to be 2 of 8 positively charged residues located within the heparin pentasaccharide binding site and to be critical for its tight binding through ionic interactions (13). In contrast, Carrell and colleagues have placed arginine 132 and lysine 133 in a putative extended heparin binding region outside of the region responsible for binding to heparin pentasaccharide, based upon their x-ray structure of antithrombin (14). Although the 2.3–4.5-fold reductions in affinity for heparin pentasaccharide are real and represent 5–9% of the total binding energy, they are small in comparison with the reductions in affinity of full-length heparin to the same variants. They are also small in comparison with the reduction in affinity of pentasaccharide to a K125M variant. The latter variant showed a reduction in pentasaccharide affinity of 170-fold and clearly established lysine 125 as

![Figure 3](image1.png) **FIG. 3.** Heparin affinity of R132M and K133M antithrombin variants monitored by changes in tryptophan fluorescence as a function of added heparin. Data are represented as percentages of maximum fluorescence change (%ΔFmax). Open squares, R132M antithrombin; filled circles, K133M antithrombin. The solid lines represent the nonlinear least-squares best fits to the experimental data. The Kd values are given in Table I. A, high affinity heparin; B, heparin pentasaccharide.

![Figure 4](image2.png) **FIG. 4.** Ionic strength dependence of binding of full-length high affinity heparin to control and R132M and K133M variant antithrombins. The linear dependence of the log-log plot is as expected for ionic contributions to heparin binding. Open diamonds and solid line, R132M antithrombin; open circles and dotted/dashed line, K133M antithrombin; open squares and dashed line, control antithrombin. The lines represent best linear fits to the experimental data. Each Kd represents the average of two to four separate determinations. The reduced slope for both variants compared to control antithrombin indicates a reduction in the number of ionic interactions (Z) that contribute to heparin binding.

| Antithrombin | Pentasaccharide | High affinity heparin |
|--------------|-----------------|-----------------------|
|              | Kd              | ΔG kJ/mol | ΔΔG kJ/mol | Kd              | ΔG kJ/mol | ΔΔG kJ/mol |
| Control      | 38 ± 7          | −42.3     |         | 2.8 ± 0.2*      | −48.8     |         |
| R132M        | 89 ± 4          | −40.2     | 2.1     | 48 ± 9          | −41.7     | 7.1      |
| K133M        | 171 ± 5         | −38.6     | 3.7     | 47 ± 17         | −41.8     | 7.0      |

* Determined by extrapolation from Kd values determined at higher ionic strength and may be an underestimate of the true affinity at 0.15 I.

**TABLE I**

Heparin-antithrombin affinities for control and variant antithrombins at I 0.15

Kd values are given as the mean and range of values obtained from a minimum of three separate full titrations.
TABLE II

Rates of inhibition of thrombin and factor Xa by control and variant antithrombins (mM⁻¹ s⁻¹)

| Antithrombin          | Thrombin   | Proteinase                  |
|-----------------------|------------|-----------------------------|
|                       | −Heparin   | +HAH                        | −Heparin | +Pentasaccharide   | +HAH |
| Control               | 8.6 ± 0.1  | 10,400 ± 1100               | 2.5 ± 0.1 | 530 ± 95           | 1400⁺ |
| R132M                 | 7.7 ± 0.1  | 17,700 ± 2000               | 3.3 ± 0.1 | 460 ± 47           | 1650 ± 10 |
| K133M                 | 6.6 ± 0.1  | 15,400 ± 1400               | 3.5 ± 0.1 | 530 ± 45           | 1620 ± 27 |

⁺ From Turko et al. (17).

**Fig. 5. Increase in rate of inhibition of factor Xa by antithrombin as a function of heparin pentasaccharide concentration.** The solid line represents the best linear fit of the data. The values for $k_{cat}/K_{m}$ obtained using the slope of the best fit line and the $K_d$ values given in Table I, are summarized in Table II. A, R132M antithrombin; B, K133M antithrombin.

a very important residue in binding the high affinity heparin pentasaccharide (12). Up to 10-fold alterations in affinity of antithrombin for heparin have been shown in a number of other cases to result from changes that do not involve alteration of specific antithrombin-heparin charge interactions. For example, the removal of carbohydrate from asparagine 135 results in a 10-fold increase in pentasaccharide affinity at I 0.15 (25), whereas the presence of core fucose on the carbohydrate attached to asparagine 155 causes a 2-fold reduction in affinity (26). The small reductions in affinity for binding of pentasaccharide to the two variants therefore do not support a direct role for these residues in the pentasaccharide binding site. The larger, ionic strength-dependent reductions in affinity for full-length high affinity heparin binding to the variants are consistent with the location of arginine 132 and lysine 133 beyond the pentasaccharide binding region as part of an extended site of ionic interactions with longer chain heparin species. Since one strong or several weaker ionic interactions are thought to be responsible for the tighter binding of full-length heparin than of heparin pentasaccharide to plasma antithrombin (3), it is likely that the residues involved include arginine 132 and lysine 133.

Proteinase inhibition measurements showed that the R132M and K133M antithrombin mutations caused no change in the basal rate of inhibition of thrombin or factor Xa and no change in the ability of either heparin pentasaccharide or full-length heparin to increase maximally the rate of inhibition of factor Xa (Table II). From the proposed charge neutralization-helix extension mechanism of heparin activation, it would be expected that elimination of a charged residue involved in this activation mechanism would give an enhanced basal rate of inhibition of factor Xa, as a result of reduced ionic repulsions causing the antithrombin to adopt a conformation in the absence of heparin that partly mimics the heparin activated state. This is not the case with either the R132M or K133M variants, which implies that neither arginine 132 nor lysine 133 is involved in the heparin activation mechanism. This is consistent with our conclusion of their lack of involvement in pentasaccharide binding. In contrast, our previous studies on a K125M antithrombin variant found an approximately 3-fold increase in the basal rate of factor Xa inhibition (12), suggesting that the conformation of this variant may indeed partly mimic that of heparin-activated antithrombin as a result of removal of a positive charge from the heparin binding site. Thus, whereas the specific helix-extension activation mechanism through charge neutralization proposed by van Boeckel and colleagues may not be correct, charge neutralization per se may still be the principal means of propagating the conformational change from the heparin binding site to the reactive center region but be limited to residues within the already formed D-helix.

Although our results therefore do not support this part of the mechanism of heparin activation of antithrombin, we have recently found evidence in favor of the second part of the proposed activation mechanism, namely the expulsion of reactive center residues P15 and P14 from β-sheet A as the means of altering the conformation of the residues in the immediate vicinity of the scissile bond. From studies on a P14 S→W antithrombin variant, we showed that, in contrast to other serpins, the P14 residue is buried in native antithrombin but becomes solvent exposed upon heparin binding, consistent with expulsion of this residue from β-sheet A (27).

Finally, although there is evidence for the involvement of arginine 132 and lysine 133 in binding long chain heparin species through additional ionic interactions, the absence of either charged residue does not appear to affect the ability of long chain heparin to bridge between antithrombin and thrombin to give a ternary complex in which the rate at which thrombin is inhibited by antithrombin is greatly accelerated (Table II). The only major role of these two charged residues thus appears to be one of increased affinity for long chain heparin species, without any critical role in either conformational change-dependent or bridging mechanisms of heparin acceleration of proteinase inhibition.

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