Requirement of Lysine Residues Outside of the Proposed Pentasaccharide Binding Region for High Affinity Heparin Binding and Activation of Human Antithrombin III*

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Variant forms of human antithrombin III with glutamate or threonine substitutions at Lys\textsuperscript{114}, Lys\textsuperscript{125}, Lys\textsuperscript{133}, Lys\textsuperscript{136}, and Lys\textsuperscript{139} were expressed in insect cells to evaluate their roles in heparin binding and activation. Recombinant native ATIII and all of the variants had very similar second order rate constants for thrombin inhibition in the absence of heparin, ranging from 1.13 × 10\textsuperscript{8} M\textsuperscript{-1} min\textsuperscript{-1} to 1.66 × 10\textsuperscript{8} M\textsuperscript{-1} min\textsuperscript{-1}. Direct binding studies using \textsuperscript{125}I-fluoresceinamine-heparin yielded a K\textsubscript{d} of 6 nM for the recombinant native ATIII and K\textsubscript{136}T, whereas K\textsubscript{114}Q and K\textsubscript{139}Q bound heparin so poorly that a K\textsubscript{d} could not be determined. K\textsubscript{125}Q had a moderately reduced affinity. Heparin binding affinity correlated directly with heparin cofactor activity. Recombinant native ATIII was nearly identical to plasma-purified ATIII, whereas K\textsubscript{114}Q and K\textsubscript{139}Q were severely impaired in heparin cofactor activity. K\textsubscript{125}Q and K\textsubscript{136}T were only slightly impaired. Based on these data, Lys\textsuperscript{114} and Lys\textsuperscript{139}, which are outside of the putative pentasaccharide binding site, play pivotal roles in the high affinity binding of heparin to ATIII and the activation of thrombin inhibitory activity.

The D-helix and adjacent regions have been widely implicated to play an important role in the heparin cofactor activity of human antithrombin III (1–4). Several positively charged amino acids in this region, including Lys\textsuperscript{114}, Lys\textsuperscript{125}, Arg\textsuperscript{129}, and Arg\textsuperscript{132}, Lys\textsuperscript{133}, Lys\textsuperscript{136}, and Lys\textsuperscript{139}, align and present a region of high positive charge density (5). This region is believed to represent the primary binding site in ATIII\textsuperscript{1} for heparin, presumably through coordinate bonds formed with sulfate groups in the linear heparin chain. Although the binding of heparin to ATIII is a requisite step in the activation process, it is not known whether the binding of heparin to the D-helix is sufficient for activation or if other residues away from the helix may be required to achieve the conformational change that leads to the activated form of ATIII. The close proximity of the lysine and arginine residues in the D-helix and adjacent regions in the intact protein would suggest that it is not possible for all of them to play roles in heparin binding, given the distribution of sulfate groups in the heparin chain (6). The first step in understanding the activation process is thus to define the role(s) of individual residues in the D-helix region, which represents at least the initial heparin binding site in ATIII.

Naturally occurring variants of ATIII have facilitated defining the precise role of two residues in the D-helix region; Arg\textsuperscript{47}, which is in the A-helix but lies in close proximity to the amino end of the D-helix in the native protein (7–10), and Arg\textsuperscript{129}, which lies near the carboxyl end of the D-helix (11). In both cases the variant proteins display reduced binding to heparin-Sepharose and reduced heparin cofactor activity. Most of the information on the roles of other residues has come from chemical modification studies. Modification of Lys\textsuperscript{125} with pyridoxal-5'-phosphate abolishes binding to heparin-Sepharose (12, 13). The effect on heparin cofactor activity was not evaluated in those studies. Several other residues including Lys\textsuperscript{107}, Lys\textsuperscript{114}, Lys\textsuperscript{133}, Lys\textsuperscript{136}, and Lys\textsuperscript{139} have been similarly implicated by chemical modification or protection from chemical modification in the presence of heparin. These studies are limited in interpretation, however, because two or more residues were modified simultaneously (14–16). Recently ATIII was expressed in baby hamster kidney cells (17, 18). The data from these studies confirmed that Lys\textsuperscript{125} is an important residue in heparin binding and activation of ATIII, whereas Arg\textsuperscript{132} and Lys\textsuperscript{133} are at least important in heparin binding. However, several other positively charged residues, including Lys\textsuperscript{290}, Lys\textsuperscript{294}, and Lys\textsuperscript{297}, which are well outside of the proposed heparin binding region, were found to be unimportant.

Several fluorescent enhancement and \textsuperscript{1}H-NMR studies (19–23) along with the crystal structure of a dimerized form of ATIII (24) have determined the precise binding pocket and conformational contribution by the heparin pentasaccharide in ATIII. It has been proposed that because the pentasaccharide does not activate ATIII toward thrombin to the same extent as longer chain heparins, heparin must play a bridging role by bringing the two molecules in proximity of one another (25–30). In the present study we show that two residues outside of the pentasaccharide binding pocket are important for heparin binding and activation of ATIII toward thrombin, possibly leading to a new model for the role of longer chain heparins. Lys\textsuperscript{114}, Lys\textsuperscript{125}, Lys\textsuperscript{133}, Lys\textsuperscript{136}, and Lys\textsuperscript{139} were independently replaced with either glutamine or threonine (Lys\textsuperscript{136}), and the variant ATIII proteins were expressed, along with recombinant native ATIII, in insect cells using a baculovirus-driven expression system. In the absence of heparin, the recombinant native ATIII and all of the variants had similar second order rate constants and formed SDS-PAGE-resistant complexes with thrombin at nearly identical levels. In contrast, the variants had heparin cofactor activities that varied when compared with the recombinant native ATIII. Although the K\textsubscript{136}T and the K\textsubscript{125}Q variants differed by about a factor of five to six, the K\textsubscript{114}Q and the K\textsubscript{139}Q variants were catastrophic, having a

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1 The abbreviations used are: ATIII, antithrombin III; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; \textsuperscript{125}I-F-heparin, \textsuperscript{125}I-fluoresceinamine heparin.
10–20-fold decrease in their rate of thrombin inhibition in the presence of heparin. Direct hepatic binding studies revealed that the recombinant native ATIII bound heparin with a $K_d$ of 6 nM, as did the K136T variant. However, affinity of the K125Q variant for heparin decreased 2-fold, yielding a $K_d$ of approximately 12 nM, whereas the K114Q and K139Q variants bound heparin so poorly that $K_a$ values could not be determined. These data define, for the first time, an important role for Lys$^{124}$ and Lys$^{139}$ in the heparin cofactor activity of ATIII by demonstrating that they are essential for hepatic binding despite the fact that the residues are outside of the pentaasparagine binding pocket. The residues may serve to align the longer chain heparins in a proper orientation so that they align properly as a template for thrombin, or they may serve to stabilize ATIII in its active conformation when heparin is present.

EXPERIMENTAL PROCEDURES

Materials—The ATIII cDNA (pBTIII-113) cloned into pBR322 was a generous gift from Dr. Savio Woo (Howard Hughes Medical Institute, Houston, TX). Sodium iodide was purchased from Amersham Corp. Electrophoresis reagents were from Bio-Rad. Autoradiography supplies were obtained from Kodak. Sf9 cells were purchased from American Type Culture Collection (Rockville, MD). High 5 cells were purchased from Invitrogen Corp. BaculoGold was purchased from Pharmergen (San Diego, CA). The pVL 1993 vector was a gift from Dr. Max D. Summers, Texas A&M (College Station, TX). Tissue culture reagents were from j) RH Scientific and Life Technologies, Inc. Oligonucleotides were synthesized by Integrated DNA Technologies. All other common biochemical reagents were purchased from Calbiochem, Sigma, or Boehringer Mannheim.

Generation and Expression of Recombinant ATIII—Site-directed mutagenesis was performed using polymerase chain reaction (PCR) (31). Four oligonucleotides were required to generate each mutant. Two were ATIII cassette oligos designated ATIII cassette antisense oligo and ATIII cassette sense oligo that flanked the region where nucleotide changes were to be introduced and two within this region contained the sequence with the desired base changes. Two primary PCR reactions were required. One reaction contained ATIII cassette sense oligo with the mutant sense oligo, and the second contained ATIII cassette antisense oligo with the mutant anti-sense oligo. The sequences of the mutant sense and antisense oligos are as follows from 5′ to 3′: K114Q, GACACCATATCAGAGCAGCAAA- CATCCTGATCAT and CTGATCATGTTGCTGATGATGTTGT; K125Q, ACTTCTTTTGGCAGCTGACTGCGAC and GTCTG-GCTAGGAGGAGGGAGGAGGTATGTATTCTGATGATGTCTGGGAGGGAGGGAGGAGGATTTG, respectively. Four oligonucleotides were subcloned into pGem for sequencing by the Sanger dideoxy method described above.

Radioiodinations—Thrombin and F-heparin were radioiodinated as described previously (32, 37). The specific activities were 15,000 and 70,000 cpm/ng for thrombin and heparin, respectively.

Analysis of Heparin Binding to ATIII Variants—The colorimetric substrate Chromozym-Th was used to determine second order rate constants for thrombin inhibition by the recombinant native ATIII and the ATIII variants (11). Various concentrations of ATIII with or without heparin were incubated for the indicated times with a constant amount of thrombin (20 nM) at 37 °C in PBS containing 1% polyethylene glycol. At each time point the reactions were subsampled into a Chromozym-Th solution containing 50 nM Tris, pH 8.3, and 277 nM NaCl. The colorimetric reactions were allowed to proceed for 5 min and then quenched by the addition of glacial acetic acid. Absorbance was read at 405 nm. Pseudo-first order rate constants were determined by plotting V/V0 versus time. Second order rate constants were determined by dividing the pseudo-first order rate constants by the respective ATIII concentrations (36).

Heparin Titration Assays—Recombinant native ATIII was incubated with the indicated heparin concentrations in the same buffer described above for 30 min at 37 °C. Thrombin was then added at a final concentration of 20 nM, and the reaction was allowed to proceed for 1 min. The degree of thrombin inhibition was determined using the Chromozym-Th assay described above.

RESULTS

Construction and Sequencing of ATIII Variants—Following primary and secondary PCR reactions, size verification, and restriction analysis, each variant cDNA was cloned into a pGemATIII vector for sequencing by the Sanger dideoxy method using Sequenase, version 2.0. Shown in Fig. 1 are the regions of the sequencing gels that include the codon that corresponds to Lys$^{124}$. Panel A shows the native ATIII cDNA sequence with the AAA codon that corresponds to Lys$^{124}$ in the mature protein column, the column was washed with 0.25 M Tris-HCl, 1 mM NaCl, pH 7.5, followed by elution with 0.25 M Tris-HCl containing 3.5 M MgCl2 (11, 34). The fractions were tested by dot blot analysis using an anti ATIII monoclonal antibody and 125I-labeled secondary antibody. Fractions containing ATIII were pooled and dialyzed extensively against PBS. Following concentration, the recombinant proteins were analyzed by Western blot analysis and SDS-PAGE on 10% polyacrylamide gels. Protein concentrations were determined by the method of Bradford (35) and quantitative Western blots.

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sequence (after signal sequence cleavage). Panel B shows the same codon in the K114Q cDNA with the PCR introduced change of A to C at the first position in the codon, changing the codon specificity to a Gln. The rest of the sequence was found to be identical to the native ATIII cDNA sequence, eliminating any concern that Taq polymerase introduced errors. All of the mutants were analyzed similarly to ensure the desired codon changes and the absence of any Taq polymerase errors (data not shown).

Expression and Purification of Recombinant ATIII— Plaque-purified recombinant baculoviruses harboring recombinant native ATIII and the K114Q variant cDNAs were independently used to infect High 5 insect cells. After 5 days the media were harvested, and the recombinant ATIII proteins were immunoaffinity purified (see “Experimental Procedures”). The proteins were analyzed by SDS-PAGE on 10% polyacrylamide gels before and after a 30-min preincubation with 600 ng of thrombin. Shown in lanes 1 and 3 are purified recombinant native ATIII and the K114Q variant, respectively. Lanes 2 and 4 show the same amounts of recombinant native ATIII and the K114Q variant incubated with thrombin.

**Table 1**

| Inhibitor          | Second order rate constant Kd (M⁻¹ min⁻¹) | Kd (nM) |
|--------------------|------------------------------------------|--------|
| Recombinant native ATIII | 1.45 ± 13% | 6      |
| ATIII K114Q        | 1.13 ± 8.7% | *      |
| ATIII K125Q        | 1.66 ± 15% | 12     |
| ATIII K136T        | 1.55 ± 11% | 6      |
| ATIII K139Q        | 1.39 ± 3%  | *      |

Determination of the second order rate constants for thrombin inhibition and heparin binding affinity for recombinant native ATIII and each variant

Thrombin (20 nM) was incubated at 37°C with recombinant native ATIII and the ATIII variant proteins at several different concentrations, ranging from 65 to 135 nM. Samples were taken at 10-min intervals for 40 min and assayed for residual thrombin activity using Chromozym-Th as a colorimetric substrate to generate pseudo-first order rate constants. Second order rate constants were calculated by dividing the pseudo-first order rate constants by the respective inhibitor concentrations. Disassociation constants were measured using solid phase binding assays as described under “Experimental Procedures.” The asterisks indicate an unmeasurable Kd.

Although heparin binding and activation are linked events, the amino acid residues involved in binding may not be directly involved in the activation process. Conversely, amino acid residues involved in activation may contribute little to the affinity of heparin binding. It is essential to look at both processes independently to gain a clearer understanding of the activation mechanism. To determine the effect of the substitutions at the different Lys positions, direct heparin binding studies were done using a solid phase binding assay in 96-well plates. 129I-F-heparin was added to each well at a concentration of 42 nM along with various amounts of unlabeled F-heparin. At equilibrium, free ligand was removed, and the contents of the wells were solubilized in 10% SDS and quantitated by γ counting (Fig. 3). 129I-F-heparin bound to recombinant native ATIII specifically and saturably, as demonstrated by the effective competition for binding by unlabeled F-heparin. In contrast, very little specific binding was observed with the K114Q variant, and the total binding was 7–8-fold lower than the recombinant native ATIII.

To determine the affinity of heparin for recombinant native ATIII and the variants, the binding of 129I-F-heparin was quantitated over a broad concentration range (Figs. 4A and 5). 129I-F-heparin binding to immobilized recombinant native ATIII was concentration-dependent and began to saturate between 50 and 100 nM. Binding to the immobilized K114Q variant was also saturable but was at least 7-fold below that of the recombinant native, demonstrating that the K114Q variant is severely impaired in the binding of heparin. A Scatchard transformation of the data in Fig. 4A is shown in Fig. 4B. A linear
of this line was used to calculate a straight line with a coefficient of correlation of 0.97. The slope terms of heparin binding. Lys125, as expected from previous demonstrated that the lysine at position 136 is not important in K136T, and K139Q variants (Fig. 5). The binding isotherm Kd the K125Q variant was transformed for Scatchard analysis, a studies, was involved in heparin binding. This is demonstrated with 3% bovine serum albumin in Tris-buffered saline, the wells were incubated with 42 nM of 125I-F-heparin and the indicated amounts of unlabeled F-heparin at 37 °C for 1 h. The wells were then washed with PBS three times and solubilized with 10% SDS. The amount of 125I-F-heparin bound was quantitated by γ counting.

regression analysis indicated that the points define a single straight line with a coefficient of correlation of 0.97. The slope of this line was used to calculate a K_d of 6 nM. This is in good agreement with other reported binding constants of heparin to plasma ATIII (36, 39). Because of the drastically reduced affinity of heparin for the K114Q variant, it was not possible to transform the data to determine a K_d.

The same experiment was also performed for the K125Q, K136T, and K139Q variants (Fig. 5). The binding isotherm demonstrated that the lysine at position 136 is not important in terms of heparin binding. Lys125, as expected from previous studies, was involved in heparin binding. This is demonstrated by the fact that when the data from the binding isotherm for the K125Q variant was transformed for Scatchard analysis, a K_d of approximately 12 nM was determined. This is a 2-fold lower affinity for heparin by the K125Q variant relative to recombinant native ATIII. Like the K114Q variant, the data from the binding isotherm for the K139Q variant could not be transformed into a clear Scatchard analysis. This indicates that the K139Q variant also bound heparin very poorly.

To ensure that the data observed in the Scatchard analysis above were not artifacts caused by a differential adsorption of recombinant native ATIII and the ATIII variants, the control experiment was done to measure the amount of ATIII physically immobilized in the wells. Recombinant native ATIII and the variant proteins were immobilized in a 96-well plate as described above. The relative amount of each protein bound was determined using monoclonal antibody 4D11/B10 (see “Experimental Procedures” and data not shown). Because the recombinant native ATIII and the variants were detected at the same levels, the difference in the binding isotherms is thus the result of a true difference in heparin binding and not an artifact resulting from unequal immobilization of the recombinant forms of ATIII to the wells.

In addition to demonstrating that the recombinant native ATIII produced in insect cells behaves identically to plasma-purified ATIII with respect to heparin binding, the binding isotherm data also demonstrate the presence of one predomi-
examined. $^{125}$I-Thrombin (20 nM) was reacted with increasing amounts of recombinant native ATIII or the K114Q variant for 30 min at 37 °C. The reactions were quenched by the addition of SDS-PAGE sample buffer, and the ATIII-thrombin complexes were resolved by SDS-PAGE on 10% polyacrylamide gels (Fig. 8). In both cases, increasing the ATIII concentration resulted in more complexes formed, and the relative amount of complexes observed were indistinguishable for recombinant native ATIII (Fig. 8, lanes 2–4) and the K114Q variant (Fig. 8, lanes 5–7).

We next compared the two under conditions of limited time and heparin availability. Recombinant native ATIII and the K114Q variant, both at a concentration of 65 nM, were incubated with heparin at the indicated concentrations for 30 min at 37 °C. $^{125}$I-Thrombin (20 nM) was added, and the reactions were quenched and analyzed as described above. Recombinant native ATIII was activated by heparin in a dose-dependent manner, with a half-maximal activation occurring at a heparin concentration of 2 nM (Fig. 9A). This is in excellent agreement with the $K_d$ of 6 nM determined in the Scatchard analysis. In contrast, the K114Q variant displayed no activation under these conditions, even at a 10-fold higher heparin concentration (Fig. 9B). Thus, whereas the effect of the K114Q substitution was very apparent under conditions optimized to promote thrombin inhibition, the effect is even more dramatic under conditions where heparin availability and time are limited. The latter is more likely to reflect the in vivo conditions where these two factors play an important role.

**DISCUSSION**

In the present study we have investigated independently the role(s) of several positively charged lysine residues in and around the D-helix of ATIII including Lys114, Lys125, Lys133, Lys136, and Lys139 by changing them to either glutamine or threonine (Lys136). Although other studies have implied a role for Lys114, Lys136, and Lys139, none has done so under conditions where these were the sole residue chemically modified (12–16) or protected (2). In addition, no other study has carefully evaluated the role of these lysine residues by directly measuring heparin binding or by quantitating the effect of a mutation at this position on heparin cofactor activity. The remaining positively charged residues in this region include Arg129, Arg132, Lys133, and Arg47 in the A-helix, all of which have been studied in detail in naturally occurring or genetically engineered variants. Arg47 (7–10) and Arg129 (11) appear to be important in heparin binding and activation, whereas Arg132 and Lys133 appear to be involved in heparin binding (18). In order to understand the mechanism by which ATIII is heparin-activated, each of these residues in the D-helix and adjacent regions have been evaluated independently at a detailed level to fully understand their role(s).

In the present studies glutamine or threonine was substituted at each residue studied because they are uncharged and similar in size to Lysine but still have polar character.
determine if the individual substitutions altered the ability of the recombinant ATIII variants to inhibit thrombin, the second order rate constants for recombinant native ATIII and all of the variant proteins were determined in parallel. The second order rate constants were found to be very similar and are in good agreement with other published values (36, 39). The similar levels of thrombin inhibition in the absence of heparin strongly suggests that the overall structure of the ATIII has not been severely altered by the substitutions. The effect of the substitutions on heparin activation was initially evaluated by measuring the time-dependent inactivation of thrombin in the presence and the absence of heparin. There was no effect seen for the K133Q variant, which was not expected from a previous study (18) (data not shown). However, the previous study only considered activation of ATIII by heparin toward factor Xa, not thrombin. In contrast, at a heparin concentration of 42 nM, which gave half-maximal activation of recombinant native ATIII, little or no activation of the K114Q or K139Q variants was observed. The K136T variant was much less affected. Although the K114Q and K139Q variants were slowed at least 10–20-fold, the K136Q was only about 5-fold slower. As expected from other studies (17) the K125Q variant was only about 6-fold slower in its inhibition of thrombin. In all cases, when the concentration of heparin was raised, no additional activation was seen.

To more precisely determine the cause for the loss of heparin activation, direct binding studies were performed using 125I-F-heparin. Recombinant native ATIII bound heparin specifically and saturably with a $K_d$ of 6 nM, which is in good agreement with published values (36, 41). The K125Q variant had decreased heparin affinity with a $K_d$ of about 12 nM. Although this indicates that the residue is not as critical as was proposed earlier by another group using chemical modification (12, 13), it does agree in scale with the recently published characterization of a Lys$^{125}$ variant of ATIII produced in baby hamster kidney cells (18). In contrast, the K114Q and K139Q variants bound heparin so poorly that a $K_d$ could not be determined. The simplest interpretation of these data is that removal of the
Lys114 and Lys139 are not actually part of the D-helix, and helical disruption is a possibility. However, impact on the binding of heparin. This cluster occurs on a face similar to that observed in the present study. It is not immediately clear why an individual Lys or Arg residue that is part of a very extensive charge cluster should have such a strong impact on the binding of heparin. This cluster occurs on a face of the D-helix, and helical disruption is a possibility. However, Lys\textsuperscript{114} and Lys\textsuperscript{139} are not actually part of the D-helix, and Arg\textsuperscript{129} is at the end of the D-helix (24). In addition, there is no disruption of nonheparin-activated antithrombin activity, suggesting no major structural alteration in the molecule.

Another group has identified a naturally occurring variant of ATIII with a Glu substitution at position Arg\textsuperscript{129} from a heterozygous individual, with thrombotic disorder (11). Although direct heparin binding studies were not done, it was noted that the Arg\textsuperscript{129} variant antithrombin no longer bound to heparin-Sepharose at a physiological salt concentration, and in addition, the degree of the loss of heparin activation was very small.

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Addition of longer chain heparin is thought to be mediated through a template mechanism that immobilizes ATIII and thrombin in close proximity (25–30). The present data demonstrate that longer chain heparins interact with residues outside of the pentasaccharide binding pocket, namely Lys\textsuperscript{114} and Lys\textsuperscript{139}, either to help facilitate and stabilize a full conformational change in ATIII caused by heparin or to position the heparin chain as a more efficient template.

The demonstration that monovalent antibody fragments directed against residues 137–145 partially mimic the action of heparin would lend more support for the former than the latter interpretation (4), but this area is clearly open to further investigation.

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