Lysine Residue 114 in Human Antithrombin III Is Required for Heparin Pentasaccharide-mediated Activation*

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Recombinant native antithrombin III (ATIII) and two genetic variants with glutamine substitutions at lysine residues 114 and 139 were expressed in insect cells using a baculovirus-driven expression system. The purified proteins were used to evaluate the potential role(s) of these residues in the pentasaccharide-mediated activation of ATIII. The second order rate constants for the inhibition of factor Xa by both of the genetic variants were nearly identical to those of recombinant native ATIII, indicating that the glutamine substitutions did not result in serious protein conformational changes. The glutamine substitution at lysine 139 had no effect on the pentasaccharide-mediated activation of ATIII toward factor Xa. In contrast, lysine 114 was found to be critical in the activation of ATIII toward factor Xa. No activation was observed, even at a pentasaccharide concentration 10 times higher than that required to activate recombinant native ATIII. These data are the first to demonstrate a pivotal role for lysine 114 in the pentasaccharide-mediated activation of ATIII.

The D-helix and adjacent regions in ATIII1 are important for the binding of long-chain heparin and heparin pentasaccharide and the subsequent activation of ATIII toward thrombin and factor Xa (1–4). Whereas the heparin pentasaccharide is sufficient to induce the complete activation of ATIII toward factor Xa, longer-chain heparins are required to achieve complete activation of ATIII toward thrombin (5–9). Current models also suggest that the pentasaccharide is sufficient to fully induce the conformational changes in ATIII associated with activation and that the requirement of longer heparin chains for the full activation of ATIII toward thrombin is completely attributable to active ATIII. This point remains controversial and is most directly contradicted by a study that demonstrated the activation of ATIII toward thrombin by a Fab fragment of an antibody that specifically recognized residues 138–145 in ATIII (4).

Naturally occurring genetic variants have demonstrated important roles for Arg-24 (16), Arg-47 (17–20), and Arg-129 (21) in heparin and pentasaccharide binding. In addition, recombinant ATIII expressed in baby hamster kidney cells has been used to implicate Lys-125 in both pentasaccharide and long-chain heparin binding (22). 1H NMR studies (5–9) and the crystal structure of a dimerized form of ATIII (23), along with the biochemical and genetic evidence, have led to a proposed pentasaccharide binding site that includes Arg-24, Arg-47, Lys-125, and Lys-129 (23). However, the potential roles of other positively charged residues in this region have not been ruled out. Recently a definitive role for Lys-114 and Lys-139 in long-chain heparin binding and ATIII activation was demonstrated using recombinant ATIII expressed in insect cells (24). These residues lie at opposite ends of the D-helix, outside of the proposed pentasaccharide binding pocket, precluding simultaneous binding to the pentasaccharide. This suggests the mechanistic role of these two residues in long-chain heparin activation may involve the alignment of heparin chains for template activity.

To test this directly, the pentasaccharide activation of human ATIII purified from plasma, recombinant native ATIII, and the two genetic variants, K114Q and K139Q, toward factor Xa was examined. In the absence of pentasaccharide, recombinant native ATIII, K114Q, and K139Q had second order rate constants for the inhibition of factor Xa that are in good agreement with the published values for ATIII purified from human plasma (22). Using a chromogenic assay to assess factor Xa inactivation by ATIII, titrations were done to determine the pentasaccharide concentration dependence of ATIII activation. ATIII purified from plasma, recombinant native ATIII, and the K139Q variant had very similar pentasaccharide activation curves. Activation was half-maximal at pentasaccharide concentrations of 31, 23, and 42 nM, respectively. These concentrations are in good agreement with the published $K_d$ value of 29 nM obtained utilizing tryptophan fluorescence measurements (22). Surprisingly, the pentasaccharide activation of the K114Q variant was so poor that an apparent affinity could not be determined, despite using pentasaccharide concentrations in excess of 10 times the $K_d$ (272 nM) for recombinant native ATIII. The pentasaccharide cofactor activity of K139Q toward factor Xa and thrombin was identical to recombinant native ATIII, suggesting no role for Lys-139 in pentasaccharide-mediated activation. As expected from the titration data, the pentasaccharide cofactor activity of K114Q was severely impaired. These data are the first to demonstrate that Lys-114 resides within the pentasaccharide binding pocket near the amino-terminal end of the D-helix in ATIII and is required for the pentasaccharide-mediated activation of ATIII toward factor Xa.

EXPERIMENTAL PROCEDURES

Materials—Electrophoresis reagents were from Bio-Rad. Sf9 insect cells were purchased from American Type Culture Collection (Rockville, MD). High Five insect cells were purchased from Invitrogen Corp. BaculoGold was purchased from Pharmingen (San Diego, CA). Tissue culture reagents were from JRH Scientific and Life Technologies, Inc. Methyl-pentaoside (ATIII-binding pentasaccharide; product SR 90107; Sanofi Labs) was a generous gift from Dr. Peter Gettins (University of California, Irvine, CA). The abbreviations used are: ATIII, antithrombin III; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HAH, high-affinity heparin; HAc, acetic acid; SERPIN, serine protease inhibitor.
Illinois-Chicago, Chicago, IL). Throughout the manuscript, this synthetic pentasaccharide is referred to simply as pentasaccharide. Chromozym X and Chromozym TH were from Boehringer Mannheim. Human thrombin and human factor Xa were purchased from Calbiochem. Na125I was purchased from Amersham. All other common biochemical reagents were purchased from Sigma.

**Generation and Expression of Recombinant ATIII**—Specific individual point mutations in the cDNA of human ATIII were generated by overlapping polymerase chain reaction. The generation, expression, and purification of the K114Q and K139Q variants have been described in detail elsewhere (24). The purity of the proteins is also documented in the same report (24). The only difference in the procedures utilized in the present studies is that the ATIII was eluted from the immunoaffinity column with 0.2 M glycine HCl (pH 2.7) in 0.15 M NaCl (25). All of the recombinant forms of ATIII, including the recombinant native ATIII, were purified using this same scheme.

**Determination of ATIII Activity**—Each of the purified recombinant proteins was quantified for total protein content by the method of Bradford (26). The activity of each of the recombinant proteins was calculated using a human ATIII purified from human plasma (27) as a reference standard. A standard curve was generated by incubating known amounts of the reference standard ATIII, ranging from 3.44 to 17.2 nM, with a constant amount of thrombin (8 nM) for 40 min at 37 °C. The reactions were done in a total volume of 100 μl of PBS/0.1% bovine serum albumin. The reactions were chilled to 4 °C, and Chromozym TH was added to a final concentration of 10 nM. The reactions were brought to room temperature, and color development was allowed to proceed for 5 min, followed by the addition of an equal volume of 1 M HAc to stop the reactions. Substrate turnover was quantified by absorbance measurements at 405 nm. Parallel reactions were done identically with known amounts of the purified ATIII recombinant proteins. Active ATIII concentrations in each of the purified recombinant ATIII preparations were determined by reference to the standard curve generated using plasma ATIII. The percentage of activity of each of the recombinant proteins was calculated by dividing the active ATIII concentration by the total ATIII concentration.

**Kinetics of Factor Xa Inhibition**—Recombinant native ATIII and each of the ATIII variants were incubated at a concentration of 140 nM with factor Xa (20 nM) for various amounts of time at 37 °C. At the appropriate times the reactions were quenched by the addition of Chromozym X. After a 3-min color development the reactions were stopped by the addition of Chromozym X, and after 3 min the reactions were stopped by the addition of 1 M HAc. Pseudo-first order rate constants were generated by plotting log V/Vo versus time. Second order rates were generated by dividing the pseudo-first order rate constants by the inhibitor concentration (28).

**Concentration Dependence of Pentasaccharide Activation**—ATIII purified from human plasma, recombinant native ATIII, and each of the ATIII variants at a concentration of 65 nM were incubated with increasing concentrations of pentasaccharide at 37 °C for 30 min. Factor Xa was then added and a final concentration of 20 nM, and the reactions were allowed to proceed for 2 min. The reactions were quenched by the addition of Chromozym X, and after 3 min the reactions were stopped by the addition of 1 M HAc. Apo active versus pentasaccharide concentration was plotted to determine the relative affinity of recombinant native ATIII and each of the variants for pentasaccharide.

**Radiolization of Thrombin**—Human thrombin was radiiodinated as described previously (4). Specific activities of individual preparations ranged from 10,000 to 23,000 cpm/μg.

**Heparin Activation of Recombinant ATIII in Insect Cell Media**—Insect cells were infected with baculovirus constructs harboring recombinant native ATIII and each of the variants. Five days later the media were harvested and clarified as described previously (24). The concentration of recombinant ATIII in each of the media was determined by quantitative Western blot analysis using a mouse monoclonal anti-ATIII antibody. Matched amounts of recombinant native ATIII (5 μg; 3.0 μl of media) and the K114Q variant (5 μg; 3.7 μl of media) were added to PBS in a final volume of 40 μl. High-affinity heparin was added to various concentrations, ranging from 0.02 to 83.0 nM, and the reactions were incubated for 30 min at 37 °C. 125I-Thrombin (1.7 nM) was added and allowed to react for 30 s, followed by the addition of SDS-PAGE sample buffer to quench the reaction. 125I-Thrombin-ATIII complexes were resolved from free 125I-thrombin by SDS-PAGE on 10% polyacrylamide gels (29). The radioactivity in the 125I-thrombin-ATIII complex bands was quantified by image analysis on a Bio-Rad GS250 molecular imager and expressed in terms of pixel density units.

**Pentasaccharide Cofactor Activity of Recombinant Native ATIII and the ATIII Variants**—Recombinant native ATIII and each of the ATIII variants at a concentration of 65 nM were incubated for 30 min at 37 °C with 23 nM pentasaccharide, which corresponds to the apparent Kd of pentasaccharide for recombinant native ATIII. Factor Xa was added to a final concentration of 20 nM, and the reactions were allowed to proceed for the indicated times. Residual factor Xa was measured as described above.

**RESULTS**

Immunofinity-purified recombinant ATIII is recovered in an active form. The purification scheme for recombinant ATIII expressed in insect cells in the present studies differs in one step from our previously published scheme (24). In our previous studies ATIII was bound to an affinity column consisting of anti-ATIII IgG coupled to Sepharose, followed by elution with 3.5 M MgCl2 buffered to pH 7.0 with 0.25 M Tris. In the present studies, ATIII was eluted with 0.2 M glycine HCl in 0.15 M NaCl (pH 2.7) at 4 °C, as described previously for the purification of a related SERPIN, protease nexin 1 (25). The eluate was collected directly into 3.0 M Tris-HCl in 0.15 M NaCl, pH 8.8, to immediately neutralize the acidic conditions. To determine if the brief exposure to the acidic conditions adversely affected the activity of the ATIII, titration experiments measuring thrombin inactivation were done using known concentrations of human ATIII, purified from plasma, as a standard. Using the known ATIII concentrations of each of the recombinant preparations, various amounts were added in parallel to the ATIII standard to determine the active recombinant ATIII concentrations. The percentage of activity of each recombinant ATIII was calculated by dividing the concentration of active recombinant ATIII by the total ATIII concentration.

**Kinetics of Factor Xa Inhibition** by Recombinant Native ATIII and the K114Q and K139Q Variants—To control for potential effects of the introduced mutations on the gross structure of the ATIII genetic variants, second order rate constants for the inhibition of factor Xa in the absence of heparin or pentasaccharide were determined. Recombinant native ATIII, K114Q, and K139Q were each incubated with factor Xa for various amounts of time. Residual factor Xa activity was quantified using the colorimetric substrate Chromozym X. Pseudo-first order rate constants were determined by plotting log V/V0 versus time and calculating the slope. The second order rate constants were calculated by dividing the pseudo-first order rate constants by the inhibitor concentrations (28). Table II shows the second order rate constants for the inhibition of factor Xa by recombinant native ATIII and the K114Q and K139Q genetic variants in the absence of pentasaccharide. The

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**TABLE I**

Characterization of the activity of recombinant native ATIII and K114Q and K139Q genetic variants

| ATIII          | Protein (μg/ml) | Percent active |
|----------------|----------------|---------------|
| Native ATIII   | 11.6           | 86.6%         |
| K114Q          | 11.0           | 81.1%         |
| K139Q          | 10.6           | 92.2%         |

Each of the recombinant forms of ATIII was purified as described under “Experimental Procedures.” Protein concentrations were determined by the method of Bradford (26). To determine the activity of each of the recombinant forms, a standard curve for thrombin inactivation was generated using known amounts of ATIII purified from human plasma. Various amounts of the recombinant proteins were assayed in parallel to determine the concentration of active ATIII in each of the preparations. The active concentrations were divided by the total protein concentration to determine the percentage of active ATIII.
values varied only slightly between each of the forms of ATIII and ranged from $1.20 \times 10^5$ to $1.46 \times 10^5$ for recombinant native ATIII and K139Q variant. These values are in good agreement with other published second order rate constants for the inhibition of factor Xa by ATIII and recombinant native ATIII in the absence of heparin or pentasaccharide.

**Table II**

| Inhibitor                        | Second order rate constant $(M^{-1} \text{min}^{-1} \times 10^9)$ |
|----------------------------------|---------------------------------------------------------------|
| Recombinant native ATIII         | 1.20 ± 0.13                                                  |
| ATIII variant K114Q              | 1.15 ± 0.13                                                  |
| ATIII variant K139Q              | 1.46 ± 0.08                                                  |

This dramatic loss of activation strongly suggests that Lys-114 plays a pivotal role in the pentasaccharide binding site. The apparent affinity of the K139Q variant was comparable to that of recombinant native ATIII, indicating that Lysine 139 has no role in the activation of ATIII toward factor Xa by the pentasaccharide.

**Figure 1. Titration of the pentasaccharide activation of plasma ATIII, recombinant native ATIII, and the K114Q and K139Q genetic variants toward factor Xa.**

- Each of the forms of ATIII at a final concentration of 65 nM was incubated with pentasaccharide at the indicated concentrations for 30 min at 37 °C. Factor Xa was then added to a final concentration of 20 nM, and the inhibitory reactions were allowed to proceed for 2 min. The reactions were quenched by the addition of Chromozym substrate, and color developments were allowed to proceed for an additional 3 min. Color development was stopped by the addition of 1 M HAc, and substrate turnover was quantified by absorbance spectroscopy at 405 nm. Plasma ATIII, □: recombinant native ATIII, ●: ATIII variant K139Q, ■: ATIII variant K114Q, ○.

The Factor Xa Pentasaccharide Cofactor Activity of the K114Q Variant Is Markedly Impaired—To evaluate the extent to which the substitutions at Lys-114 and Lys-139 affected pentasaccharide cofactor activity, a time course of factor Xa inhibition was done at a pentasaccharide concentration of 23 nM with recombinant native ATIII and each of the genetic variants (Fig. 2). In each case, the ATIII concentration (65 nM) was three times the factor Xa concentration (20 nM). At the indicated times, samples were quenched by the addition of Chromozym X substrate and quantified for residual factor Xa activity as described under “Experimental Procedures.” The inhibitory activity of K114Q in the absence of added pentasaccharide is shown for comparison. As expected from the results obtained in the titration experiments (Fig. 1), the pentasaccharide cofactor activity of the K139Q variant paralleled that of the recombinant native ATIII. However, the pentasaccharide cofactor activity of the K114Q variant was markedly impaired at this concentration of pentasaccharide throughout the 20-min time course. These data further substantiate the role of Lys-114 in the pentasaccharide activation of ATIII.

**The Loss of Pentasaccharide Cofactor Activity in the K114Q Variant Is Not a Purification Artifact**—Previous studies using bovine antithrombin have shown that exposure of antithrombin to acidic conditions results in irreversible partial loss of...
heparin cofactor activity (30). Although the present studies used a purification procedure that exposed the ATIII to acidic conditions, the conditions differ considerably from those in the studies with bovine antithrombin (30). The acidic exposure in the present studies was brief, was at 4 °C, and has been used successfully in the immunoaffinity purification of PN1 (25). However, given the importance of this point in the present studies, we sought to directly demonstrate that the loss of heparin cofactor activity in the K114Q variant was not a result of the purification procedure. To accomplish this, heparin titration experiments were done using recombinant ATIII in media harvested from insect cells before purification. Recombinant native ATIII and the K114Q variant, each at a concentration of 2.2 nM, were preincubated with increasing concentrations of high-affinity heparin (HAH) ranging from .02 to 83 nM for 30 min at 37 °C. 125I-Thrombin (1.7 nM) was added, and the reactions were continued for 30 s. The reactions were quenched by the addition of an equal volume of 2x SDS-PAGE sample buffer. 125I-Thrombin-ATIII complexes were resolved by SDS-PAGE on 10% polyacrylamide gels and analyzed on a Bio-Rad GS 250 molecular imager. The data is expressed in pixel density units. At the largest pixel density unit value, approximately 40% of the added thrombin is in complex with ATIII.

**DISCUSSION**

In a previous study we identified Lys-114 and Lys-139 as absolute requirements for the activation of ATIII toward thrombin by long-chain heparin (24). One of the key observations in these studies was the nearly complete inability of these two variants to bind long-chain heparin. Because both Lys-114 and Lys-139 were thought to lie outside the proposed pentasaccharide binding site (23), which is hypothesized to mediate the conformational changes associated with activation, the precise biochemical reason for the requirement of these residues in the activation of ATIII by long-chain heparin was not immediately obvious.

In the present studies we observed that the substitution of a glutamine residue at the position of Lys-114 severely impaired the pentasaccharide activation of ATIII toward factor Xa. In contrast, the substitution of a glutamine at the position of Lys-139 had no effect on the pentasaccharide-mediated activation of ATIII toward factor Xa. The normal second order rate constants of these genetic variants for the inhibition of factor Xa in the absence of heparin or the pentasaccharide argue strongly against these results being artifactual. Additionally, because the effect of the substitution at Lys-114 was apparent with our previously published results (24) as well as those of others (22, 28). In contrast, the K114Q variant showed no activation at this concentration and was only minimally activated at a much higher HAH concentration (83 nM). These results clearly demonstrate that the loss of cofactor activity is not an artifact of the purification because it was apparent before exposure to the purification conditions.
in crude insect cell supernatants before ATIII purification, it is not likely to be due to a problem of differential stability to the purification conditions.

The identification of amino acid residues in and around the D-helix of ATIII that are thought to define the pentasaccharide binding site has been derived from both biochemical and genetic evidence. Using these data, in combination with the recently determined crystal structure, a model has been proposed to describe the pentasaccharide binding site in ATIII. The proposed site includes Arg-129 and Lys-125, both located in the D-helix, and Arg-47 in the A-helix. Indeed, there is an overwhelming amount of genetic evidence to support a model that includes these residues. This model also includes Arg-24 in the pentasaccharide binding site, although the genetic evidence to support this, a single point mutation of Arg to Cys, is somewhat less convincing (16). Based on the evidence obtained in the present studies, we propose a refinement in the model to include Lys-114 as a key residue in the ATIII pentasaccharide binding site (Fig. 4). Lys-114 lies just amino-terminal to the start of the D-helix and aligns with Lys-125 and Arg-129 in the D-helix. In contrast, Lys-139, which is well off the D-helix, is clearly outside of the pentasaccharide site because a Gln substitution at this position had no effect on the pentasaccharide activation of ATIII toward factor Xa.

FIG. 4. Pentasaccharide binding site in human antithrombin III. The coordinates for the crystal structure of inhibitory ATIII derived from dimeric antithrombin (23) were obtained from the Brookhaven Protein Databank file 1ANT. The figure shown was generated using the modeling program Setor, so that the helices could be represented as tube structures for easy orientation. The positions of the residues that have been implicated to reside within the pentasaccharide binding pocket of ATIII are indicated by black dots. Based on the data presented in the present report, we have included Lys-114, underlined, as a member of the pentasaccharide binding pocket. The position of Lys-139, which is required for the binding of long-chain heparin but not pentasaccharide, is also indicated. The position of the cleavage site at the reactive center is indicated by an asterisk. The three dashed lines indicate three short regions of poor electron density where coordinates were not available. These include the amino-terminal residues 1–12, 30–42, and 378–382.

ACKNOWLEDGMENTS—Dr. Mary Knauer is gratefully acknowledged for generation of the ATIII pentasaccharide binding model using the modeling program Setor and also for her critical evaluation of the manuscript.

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