Helix D Elongation and Allosteric Activation of Antithrombin*

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Antithrombin requires allosteric activation by heparin for efficient inhibition of its target protease, factor Xa. A pentasaccharide sequence found in heparin activates antithrombin by inducing conformational changes that affect the reactive center of the inhibitor resulting in optimal recognition by factor Xa. The mechanism of transmission of the activating conformational change from the heparin-binding region to the reactive center loop remains unresolved. To investigate the role of helix D elongation in the allosteric activation of antithrombin, we substituted a proline residue for Lys133. Heparin binding affinity was reduced by 25-fold for the proline variant compared with the control, and a significant decrease in the associated intrinsic fluorescence enhancement was also observed. Rapid kinetic studies revealed that the main reason for the reduced affinity for heparin was an increase in the rate of the reverse conformational change step. The pentasaccharide-accelerated rate of factor Xa inhibition for the proline variant was 10-fold lower than control, demonstrating that the proline variant cannot be fully activated toward factor Xa. We conclude that helix D elongation is critical for the full conversion of antithrombin to its high affinity, activated state, and we propose a mechanism to explain how helix D elongation is coupled to allosteric activation.

Antithrombin, a member of the serpin family of protease inhibitors (1–4), is a key regulator of blood coagulation. Antithrombin circulates in a self-constrained inactive conformation (5–7) and requires the binding of its cofactor heparin for efficient inhibition of target proteases, factor Xa, and thrombin (Fig. 1). Heparin binding is a two-step process with an initial weak interaction followed by an activating conformational change to a high affinity state (8). A specific pentasaccharide sequence found in a fraction of heparin chains is required for tight binding and full induction of the activating conformational change in antithrombin (9), and in isolation accelerates the rate of factor Xa inhibition by ~300-fold (10). The pentasaccharide binding site was defined in 1997 with the completion of the crystallographic structure of the antithrombin-pentasaccharide complex (Fig. 1) (11). The structure confirmed predictions that the pentasaccharide interacts with a region of positively charged residues located primarily on helix D (12), however, in a more compact manner than previously expected (13), with basic residues 132, 133, and 136 not within hydrogen-bonding distances. Binding of the pentasaccharide resulted in a global conformational change in antithrombin, including secondary structural changes in the heparin binding region, expulsion of the preinserted reactive center loop hinge region from β-sheet A, closure of β-sheet A to the five-stranded form found in all other known native serpin structures, and associated tertiary structural changes (Fig. 1). In the heparin-binding region, the pentasaccharide induced a two-turn elongation of helix D at its C terminus, a new one-turn P-helix at its N terminus, and a one-turn elongation at the N terminus of helix A. Importantly, the P- and A-helix extensions are structural changes common to both latent and inhibitory molecules upon pentasaccharide binding, whereas the elongation of helix D occurs only in the inhibitory molecule (11). The resulting expulsion of the reactive center loop from β-sheet A was once thought to be the sole basis of improved inhibition of factor Xa and was thus considered to be the activating conformational change (14, 15). It has since been discovered that pentasaccharide activation of antithrombin inhibition of factor Xa is insensitive to the reactive center loop sequence and that determinants of allosteric activation lie outside this region (16, 17). Thus, loop expulsion either exposes exosites or allows simultaneous binding to the reactive center and to the exosites as observed in the heparin cofactor II-S195A thrombin structure (18) (JMO). In any case, it is clear that loop expulsion to the five-stranded β-sheet A form is a critical determinant of activation.

Despite the crystal structures, the mechanism behind the heparin-induced activation of antithrombin remains unclear. In 1994, based solely on the structure of native antithrombin and molecular dynamics simulations, van Boeckel et al. (13) proposed a mechanism whereby charge neutralization at the heparin binding region would cause helix D elongation, resulting in strain on strand 2A and thus the collapse of β-sheet A to the five-stranded form (4). This model was largely validated by the structure of the pentasaccharide-activated antithrombin. Heparin binding did result in expulsion of the reactive center loop and a collapse to the five-stranded form, and helix D elongation was observed. However, helix D elongation was not caused by the direct binding of residues C-terminal to helix D (Arg132, Lys133, and Lys136) to the pentasaccharide, and thus it remained unclear as to whether helix D was elongated as the result of more distant electrostatic forces or other conformational changes in antithrombin. A modified version of the van Boeckel model was recently proposed (19), which suggested that subtle perturbations of surface electrostatics, induced by pentasaccharide binding, shift the position of an equilibrium from the native to the activated conformation. Whatever the mode of transmission, it is reasonable to hypothesize that helix D extension plays a critical part in the activating conformational change, based on the fact that helix D elongation is...
uniquely observed in the active antithrombin component of the pentasaccharide-bound crystallographic dimer (11).

Prolines have long been known as helix breakers (20). In antithrombin, two natural variants, S116P and N118P, were found associated with thrombosis due to a deficiency in heparin binding (21). The structure of antithrombin bound to the heparin pentasaccharide provides an explanation of this deficiency, since prolines at the N terminus of helix D would inhibit the formation of the P helix. We followed a similar approach to investigate the role of helix D elongation in the activating conformational change of antithrombin by mutating residue Lys133 to proline. An alanine variant was also created to serve as a neutral control. We demonstrate that helix D elongation is coupled to the activating conformational change and is crucial for trapping the equilibrium in the activated state.

EXPERIMENTAL PROCEDURES

Proteases and Heparin—Human α-thrombin and human factor Xa (predominantly α-form) were obtained from Sigma. Protease concentrations were based on active site titrations with known concentrations of plasma antithrombin. The α-methyl glycoside of a synthetic heparin pentasaccharide, corresponding to the antithrombin binding sequence in heparin, was generously provided by Maurice Petitou (Sanofi Recherche, France), as was a high affinity pentasaccharide containing an extra sulfate group. A full-length heparin containing the pentasaccharide with an average molecular weight of 8000 (26 saccharide units) was generously provided by Steve Olson (University of Illinois). Concentrations of pentasaccharide and full-length heparin were determined by stoichiometric titrations of the saccharides into plasma antithrombin solutions of known concentration.

Expression and Purification of Variant Antithrombins—All antithrombin variants were produced on the recombinant β-glycoform antithrombin template of N135Q, as described previously (7). Recombinant antithrombin was purified from serum-free growth medium of stably transfected cells by heparin-Sepharose chromatography and size exclusion on Sephacryl-G200. Two antithrombin glycoforms were isolated, having a 2-fold difference in heparin affinity, designated H and L forms (22). The high affinity (H) glycoforms for N135Q, K133A/N135Q, and K133P/N135Q were used for all experiments in this study and are referred to as N135Q, K133A, and K133P, respectively. The K133A variant eluted at an identical salt concentration to N135Q control, demonstrating that the Lys133 residue is not essential for heparin binding. The K133P variant displayed a strongly reduced heparin affinity compared with N135Q control, eluting from heparin-Sepharose at approximately ~0.6 M NaCl, compared with ~1.2 M for N135Q. Protein was concentrated to 1 mg/ml in 20 mM NaPi, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4, 0.1 M NaCl (I = 0.15) buffer, snap-frozen in liquid nitrogen, and stored at ~70 °C. To verify that all of the material maintained full activity, antithrombin and excess protease (factor Xa and thrombin) were incubated for 15 min at room temperature and run on a nonreducing 10% SDS gel. 4-(2-Aminoethyl)-benzenesulfonfonyl fluoride was added after the incubation to inactivate excess protease. Protein bands were visualized with Coomassie Brilliant Blue. The three purified recombinant antithrombin variants were determined to be homogeneous by analysis on SDS-PAGE. In the absence of protease, all variants migrated as a single band with the same mobility as plasma β-antithrombin. All variants were able to form the characteristic high molecular weight SDS-stable complex with thrombin and factor Xa, and in the presence of excess protease all of the native band disappeared for each variant (data not shown), indicating the absence of a significant fraction of unreactive, latent material.

Stoichiometry of Inhibition—Stoichiometries of inhibition were determined in the presence and in the absence of heparin by incubating antithrombin (10–100 nM) with 100 nM factor Xa or thrombin, as described previously (23). In the presence of heparin, 100 nM pentasaccharide or full-length heparin was used. Substrate hydrolysis was measured by change in absorbance at 405 nm on a Kontron spectrophotometer using chromogenic substrate S-2222 or S-2238 for factor Xa and thrombin, respectively. All reactions were carried out in buffer containing 20 mM NaPi, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4, and 0.1 M NaCl (I = 0.15) in PEG 20,000-coated cuvettes. The Lys133 variants reacted with stoichiometries comparable with the N135Q antithrombin control, which were close to 1 in all cases. The lack of differences in SI for the variants demonstrates that the mutations did not affect the inhibitory mechanism of antithrombin and further verified the absence of a significant fraction of the latent conformer.
Dissociation Constants—The equilibrium dissociation constants for the heparin-antithrombin interaction were determined by monitoring the increase in intrinsic fluorescence with increasing amounts of heparin, as described previously (23). A solution of antithrombin (7.5 nm to 1 μM) was titrated with small additions of concentrated heparin solution for minimal dilution. Solutions were made in buffers containing 20 mM NaP04, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4 (I = 0.15). Measurements were made on a PerkinElmer Life Sciences 50B spectrophotometer. The pseudo-first-order rate constant (kobs) was obtained by dividing kobs by the initial antithrombin concentration.

Rapid Kinetic Analysis—Heparin binds antithrombin by a mechanism with two kinetically resolvable steps illustrated in Scheme 1, where an initial rapid equilibrium, Kd, between antithrombin, AT, and heparin containing the pentasaccharide sequence, H, leads to a weak complex, AT·H, followed by a rapid conformational change in antithrombin via k2 to a high affinity, highly fluorescent complex, AT·H.

The overall dissociation constant is described by $K_d = K_2[k_2 + k_{obs}]$, with $k_2$ being rate-limiting for the reverse reaction and $k_{obs}$ indistinguishable from $k_{app}$.

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Stopped-flow analysis was conducted to determine the individual rate constants for oligosaccharide binding to the antithrombin variants. The reactions were measured under pseudo-first-order conditions in which the oligosaccharide/antithrombin molar ratio was at least 5:1, as described previously (8, 23). Measurements were made on an Applied Photophysics stopped-flow fluorometer in I = 0.15 buffer. The interaction was monitored from the increase in intrinsic fluorescence with an excitation wavelength set to 280 nm and an emission filter for wavelengths above 310 nm. The acquired fluorescence traces contained 400 points, and the duration ranged from 2 to 0.05 s, depending on the rate of the reaction. Traces representing the average of about 10 acquisitions were fit to a single exponential yielding the pseudo-first-order rate constant $k_{obs}$. $k_{obs}$ values were plotted against the initial concentration of oligosaccharide, [H]0, and fitted to Equation 2,

$$k_{obs} = k_2 + k_{obs}$$

Equilibrium dissociation constants and fluorescence enhancements (I = 0.15) are tabulated in Table I.

TABLE I  
Equilibrium dissociation constants and fluorescence enhancements (I = 0.15)  

|       | Fmax | Km  | Fmax | Km |
|-------|------|-----|------|----|
|       | %    | nm  | %    | nm |
| N135Q | 4.8 ± 1.3 | 42 ± 5 | 2.1 ± 0.1 | 39 ± 2 |
| K133A | 3.2 ± 0.3 | 38 ± 1 | 4.2 ± 0.7 | 37 ± 1 |
| K133P | 79 ± 9 | 10 ± 1 | 69 ± 1.5 | 16 ± 2 |

* H5 is the physiological pentasaccharide.

** HAH represents high affinity heparin of an average of 26 saccharide units containing the pentasaccharide.

† HS is the high affinity pentasaccharide analogue containing an extra sulfate group.

* ND, not determined.

RESULTS

Heparin Affinity—Heparin affinity was determined by titrating heparin into solutions of antithrombin and monitoring changes in tryptophan fluorescence, as described previously (12). The dissociation constants of pentasaccharide and heparin binding to all three antithrombin variants were determined (Fig. 2 and Table I). At physiological ionic strength (I = 0.15), the control N135Q antithrombin bound to the pentasaccharide with a $K_d$ of 4.8 nm, similar to previously published data (25). The K133A antithrombin variant displayed a $K_d$ similar to N135Q (3.2 nm) consistent with Lys133 not being involved directly in pentasaccharide binding, as concluded by a previous study (26). A 25-fold increase in $K_d$ to 79 nm was observed for K133P.
K133P when compared with the control K133A. Full-length high affinity heparin bound to N135Q antithrombin almost 2-fold more tightly than the pentasaccharide at I = 0.15; however, K133A and K133P bound with affinities similar to those with the pentasaccharide alone. This result is consistent with the involvement of Lys335 in binding to long chain heparin molecules outside the pentasaccharide region (26).

Magnitude of Fluorescence Enhancement—Heparin binding to antithrombin typically results in a 40% fluorescence enhancement as the result of an alteration in the environment of tryptophan residues. Antithrombin contains four tryptophan residues, at positions 49, 189, 225, and 307, with tryptophans enhancement as the result of an alteration in the environment of antithrombin typically results in a 40% fluorescence enhancement. The environments of the other tryptophans are influenced by the global structural changes associated with conversion to the high affinity, activated conformation, consistent with the basal rate of thrombin inhibition being insensitive to antithrombin conformation and with the bridging mechanism being unperturbed.

Rapid Kinetic Studies—Rapid kinetics studies were performed for the antithrombin variants in order to determine which of the binding steps in Scheme 1 were affected. The hypothesis that helix D elongation is a critical part of the activating conformational change would lead to the prediction that either $k_2$ or $k_{-2}$ (the forward or reverse conformational change steps) would be affected. Fig. 3, a–c, shows the nonlinear fits at high pentasaccharide concentrations for N135Q, K133A, and K133P, respectively. Insets are linear fits at low concentrations of pentasaccharide. The slope of linear fits represents the overall second-order association rate constant, $k_{on}$, and the y intercept corresponds to the rate of the reverse conformational change step, $k_{off}$, which is indistinguishable from the overall dissociation rate, $k_{off}$. These data are given in Table III with calculated $K_a$ values determined from the values of $k_{on}$ and $k_{off}$. The agreement between observed and calculated $K_a$ values confirms the accuracy of the data obtained at low pentasaccharide concentrations despite the high relative error in extrapolation to 0 M pentasaccharide. The most striking difference observed from these rapid kinetic studies is a minimal 10-fold increase in $k_{-2}$ from $-1 \text{s}^{-1}$ for the controls to 9.7 ± 2.8 s$^{-1}$ for the proline variant. For both the alanine and proline variants, there is an increase in the $k_{on}$ from 74 ± 1 for N135Q to 187 ± 6 and 125 ± 4 for K133A and K133P, respectively. This increase in $k_{on}$ for both of the Lys133 variants is primarily accounted for by the decrease in the dissociation constant of the initial binding step, $K_1$, from 8 ± 0.4 for N135Q to 2.2 ± 0.2 and 4.8 ± 0.42 for K133A and K133P, respectively, with no significant change in the forward rate of conformational change, $k_2$.

Thermal Stability and Peptide Annealing Studies—We determined the thermal stability of the proline variant and the alanine control in the absence and presence of excess high affinity pentasaccharide by monitoring the change in circular dichroism signal at 222 nm with temperature (data not shown). The melting point of the proline variant was indistinguishable from control in the absence of pentasaccharide (56 °C) and was 2 °C lower than that of the control (63 versus 65 °C) in the presence of a 5-fold excess of the high affinity pentasaccharide. This is consistent with an altered activated conformation where certain stabilizing interactions cannot be formed due to the presence of the probe at position 133. The low intrinsic fluorescent state of the K133P variant under these conditions suggests a $\beta$-sheet A conformation that is not fully closed to the five-stranded form (Fig. 4, a and b). Such a conformation would
more readily incorporate exogenous peptides derived from the sequence of the antithrombin reactive center loop. Incorporation of peptides causes antithrombin to react with thrombin as a substrate; therefore, it is possible to determine the fraction of peptide-complexed antithrombin by SDS-PAGE following incubation with excess thrombin (32). Thus, ~20% of the proline variant, as opposed to only 5% of the alanine control, was cleaved by thrombin after incubation with the peptide (Fig. 4c). It is unlikely that the increased ability to bind peptide is caused by the minimal reduction in thermal stability for the pentasaccharide-bound proline variant, since peptide annealing reactions were carried out 26 °C below the denaturation temperature. A more plausible explanation is that the pentasaccharide-bound conformation of the proline variant lacks the stabilizing hydrogen bonds between the tops of strands 3 and 5A and thus more readily accepts peptides into the position of strand 4A.

**Fig. 3.** Stopped-flow kinetics for recombinant antithrombin variants binding to the pentasaccharide. The observed rates of oligosaccharide binding detected by changes in intrinsic fluorescence are plotted versus total concentration of oligosaccharide. Nonlinear fits at high pentasaccharide concentration for N135Q (a), K133A (b), and K133P (c), are shown in the panels with linear fits at low pentasaccharide concentration in the insets.
DISCUSSION

The aim of this study was to determine the role of helix D elongation in the allosteric activation of antithrombin. We examined the effect of a proline substitution at Lys133 in the extended heparin-binding region and found that a blocking or distortion of the extension of helix D resulted in the inability to become fully activated, as measured by fluorescence enhancement and kinetics of inhibition. Rapid heparin binding studies indicated that the reason full activation was not achieved is that the conformational changes that stabilize the high affinity state were perturbed. Thus, helix D elongation is not required for the forward steps but is a major contributing factor to the stabilization of the activated state.

Such a conclusion is supported by the properties and structures of the six-stranded forms of antithrombin. Crystal structures of several β-sheet A-expanded, six-stranded forms of antithrombin have been solved, including cleaved, latent, and peptide-complexed (33–35). The dissociation constants for pentasaccharide binding to all of these conformers are around 20 μM (35) which is similar to the dissociation constant for native antithrombin preceding the activating conformational change (K1 in Scheme 1) (8). This suggests that the conformational changes responsible for the high affinity state cannot occur when β-sheet A is six-stranded or indeed that the conformational change that leads to the high affinity state is simply closure of β-sheet A to the five-stranded form. Proof that helix D elongation is conformationally linked to the closing of β-sheet A and the expulsion of the reactive center loop is provided by the structure of latent antithrombin bound to the pentasaccharide (11). Although the pentasaccharide is bound in the same position in the latent and inhibitory conformers, and therefore the latent molecule is sampling the same neutralization of basic residues in the heparin binding region, helix D is not elongated in the latent molecule, whereas the other secondary structural changes in the heparin binding region, helix A elongation and helix P formation, were common to both latent and inhibitory forms of antithrombin when bound to the pentasaccharide.

A further indication of the importance of the region C-terminal to helix D is provided by studies on variants with truncations in the loop stretching from helix D to e2A (36). Truncation of one or several amino acids starting at position 134 resulted in reduced intrinsic fluorescence enhancement, affinity for heparin, and pentasaccharide-catalyzed rate of factor Xa inhibition. The authors concluded that truncations in this region somehow decoupled heparin binding from expulsion of the reactive center loop. For a perfectly coupled equilibrium between a native, low affinity, low fluorescence, low anti-factor Xa activity state, A, and a heparin-bound, high affinity, high fluorescence, high anti-factor Xa activity state, B, the observed rate of factor Xa inhibition and the level of intrinsic fluorescence enhancement would correlate the B/A ratio (Fig. 4a). Since most of the heparin-induced fluorescence enhancement is due to the closing of β-sheet A to the five-stranded form and associated tertiary structural changes (27), a 2-fold lower fluorescence enhancement would imply either a B/A ratio 2-fold higher for the variant in the absence of heparin or a B/A ratio 2-fold lower in the presence of heparin. If the B/A ratio were 2-fold higher in the absence of heparin, the basal rate of factor Xa inhibition would be half that of the fully catalyzed rate, and if the B/A ratio in the presence of saturating heparin were 2-fold lower, then a 2-fold reduction in the catalyzed rate of factor Xa inhibition would be expected. However, whether the helix D loop is truncated (36) or perturbed by the incorporation of a proline, a 2–3-fold decrease in intrinsic fluorescence enhancement corresponds to a reduction in basal rate of factor Xa inhibition and a 10-fold decrease in the maximal pentasaccharide-catalyzed rate of factor Xa inhibition. This would suggest that the intrinsic fluorescence is decoupled from activation as the result of an altered activated conformation, B’. Thus, although the B’ state is fully populated at saturation, its intrinsic fluorescence and inhibitory activity toward factor Xa are sig-

### Table III

**Kinetic constants for pentasaccharide binding**

| Variant | kₐ | K₁ | k₂ | K₋₋₂ | K₋₋₋₂ |
|---------|----|----|----|------|------|
| N135Q   | 74.2 ± 1 | 8.0 ± 0.4 | 704 ± 9 | 0.6 ± 0.6 | 8.4 |
| K133A   | 187 ± 5.7 | 2.2 ± 0.2 | 750 ± 15 | 1.1 ± 3.5 | 5.9 |
| K133P   | 125 ± 4 | 4.8 ± 0.4 | 595 ± 10 | 9.7 ± 2.8 | 78 |

*Fig. 4. A model for the altered pentasaccharide-activated conformation. β-Sheet A (red) responds to pentasaccharide binding by expelling the partial strand 4A, composed of the hinge region of the reactive center loop (yellow) and closing to form a parallel β-sheet interaction between strands 3 and 5A as shown in a. The fluorescent quantum yield and activity toward factor Xa are dependent on the fraction of state B relative to state A. For the K133P variant (b) where helix D (cyan) elongation is perturbed, a low fluorescence, low anti-factor Xa activity prevails at saturating levels of pentasaccharide, B’. The B’ state would be of intermediate fluorescence and activity due to a partially open conformation of β-sheet A. Such a conformation will more readily accept exogenous peptides as determined by SDS-PAGE, c. lanes 1–3 are of the control K133A, and lanes 4–6 are for K133P. Unreacted material is in lanes 1 and 4. Reaction with an excess of thrombin after incubation with the peptide for 0 (lanes 2 and 5) and 12 h (lanes 3 and 6) indicates a higher level of cleaved material for the K133P variant, consistent with a greater fraction of peptide-annealed material.*
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Fig. 5. Exposure of a large hydrophobic pocket upon conformational activation of antithrombin. Native (left) and pentasaccharide-activated antithrombin (right) are shown as α-carbon worm representations for orientation (a) and as surface contours (b), colored green according to hydrophobicity. The structures of all six-stranded forms of antithrombin resemble native in the orientation of Tyr131 (yellow), which occupies a hydrophobic pocket between helix D and s2A. In the activated conformation, Tyr131 flips out, creating a large hydrophobic pocket indicated by the red oval. This figure was made using Grasp (44).

significantly reduced relative to B. A model for B’ would need to explain both properties. It is known that the fluorescent state of antithrombin is related to the state of β-sheet A, with the five-stranded form of highest quantum yield. The rate of reaction with factor Xa is also sensitive to the state of the five-stranded form and favor the activated conformation over the native, cleaved, latent, latent bound-to-pentasaccharide, and peptide-complexed states (39) of PAI-1. Cleaved (37), latent (38), and peptide-complexed (39) structures of PAI-1 have Tyr79 (Tyr102 in the unprocessed chain) oriented in the cleft between helix D and s2A, as in all six-stranded forms of antithrombin, and flipped out of the pocket in the active five-stranded form. A similar but much smaller cavity has been identified in native α1-antitrypsin (40, 41). It is possible that compounds that specifically bind to this pocket in antithrombin may stabilize the five-stranded form and favor the activated conformation over native in the absence of heparin. Studies are currently under way to investigate the role of Tyr131 in the allosteric activation of antithrombin and to characterize the binding of small organic compounds to the heparin-binding site.

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