The binding of pentasaccharide heparin to antithrombin induces a conformational change that is transmitted to the reactive center loop and increases the rate of inhibition of factor Xa by ~300-fold. The mechanism of such transmission is not known. To test the role of residues 134–137, which link helix D to β-sheet A, in this signal transduction, we created variant antithrombins in which we removed amino acids 134–137 stepwise and cumulatively. Although the deletions did not compromise the fundamental ability of antithrombin to bind to heparin or to inhibit target proteinases thrombin and factor Xa, they did largely decouple conformational changes in the heparin-binding site from conformational activation of the reactive center loop. Because the variant with only Ala134 removed was as compromised as variants with larger deletions, yet the variant with Ser137 removed was normal, we concluded that the length of the linker is less important than the precise interrelationship between residues in this region and other residues involved in conformational activation of antithrombin.

Antithrombin is a plasma protein and a member of the serpin family of proteinase inhibitors that can inhibit all of the serine proteinases involved in the blood coagulation cascade (1). Its primary physiological targets, however, are thrombin and factor Xa. In its native state antithrombin exists predominantly in a conformation in which residues P15 and P14 (positions 15 and 14 residues N-terminal of the scissile bond, which is defined as the bond between the P1 and P1′ residues) of the reactive center loop are inserted into β-sheet A (state A). In this conformation the scissile P1–P1′ bond is constrained into a conformation that is very poorly reactive with both factor Xa and thrombin (2). A second conformation exists (state B), in which residues P15 and P14 have been expelled from β-sheet A, with consequent alteration in the conformation and/or flexibility of the P1–P1′ bond (3) and 300-fold increase in the rate of reaction with factor Xa. To permit regulation of the location and rate of inhibition of factor Xa, it appears that antithrombin has evolved to require heparin as a high affinity allosteric activator that can shift the position of the equilibrium between ~100% A in the absence of heparin to ~100% B state upon heparin binding. A specific pentasaccharide sequence in heparin is required for the high affinity binding. Other sulfated polysaccharides, such as low affinity heparin or dermatan sulfate, give intermediate degrees of activation (3, 4), which can be interpreted either as resulting from intermediate positions of the equilibrium between A and B states or conversion of the A state of antithrombin into alternative conformations with different rates of reaction with factor Xa.

Although much work has previously been carried out to identify the high affinity binding site for heparin pentasaccharide, using chemical modification, site-directed mutagenesis, and studies of natural antithrombin variants, it was the solution of the crystal structure of the pentasaccharide-bound form of antithrombin (5) that first clearly defined the site. This structure, together with that of native antithrombin (6), established the conformational changes that occur within this region as a result of heparin binding. These structures show that the highly negatively charged pentasaccharide interacts specifically with basic residues on helix D (Lys125 and Arg129), helix A (Arg46 and Arg47), and the N terminus (Lys11 and Arg13). Binding furthermore induces extension of helix D at its C-terminal end and results in formation of a new one-turn helix, helix P, which interacts with heparin through residues Lys113 and Glu111. These structures also confirmed conclusions from biochemical studies (7, 8) that indicated that heparin binding resulted in expulsion of the P14 and P15 residues from β-sheet A (Fig. 1).

Despite the great advance in understanding of the mechanism of heparin activation of antithrombin that resulted from elucidation of these crystal structures, a major question remained unanswered. That is, how do the conformational changes that occur in the heparin-binding site upon binding heparin pentasaccharide result in transmission of a signal to β-sheet A that brings about expulsion of residues P15 and P14 of the reactive center loop and hence activation toward reaction with factor Xa?

On the basis of the structure of native antithrombin alone (9), it had been proposed in 1994 that the heparin pentasaccharide-binding site included Arg132 and Lys133 and that heparin binding induces a previously unstructured region of six residues, including Arg132 and Lys133, to adopt a helical conformation and thereby extend helix D. This elongation was predicted to influence the position of the outer strands of β-sheet A (strands 2 and 3) and thereby result in a displacement of P14 and P15 from the β-sheet (10). Both mutagenesis studies (11) and the more recent crystal structure of the pentasaccharide-antithrombin complex (5) showed that neither Arg132 nor Lys133 was part of the pentasaccharide-binding site. Nevertheless both of these residues were shown to be part of the extension to the helix D that is induced by heparin binding further down the helix, as originally predicted. We therefore sought to test the idea that the coupling mechanism between heparin-induced changes in the heparin-binding site and displacement of the reactive center loop from β-sheet A involves a direct effect on strand 2A. We constructed a series...
Heparin Activation of Antithrombin

Heparin Activation of Antithrombin—The active concentration of full-length antithrombin was determined by stoichiometry of inhibition using 1 μM antithrombin and 20 μM TNS
2 with full-length high-affinity heparin previously standardized against fully active plasma antithrombin, using the well-defined inflection point of the fluorescence at saturation with heparin (15). Measurements were made on an SLM 8000 spectrophotometer, exciting TNS at a wavelength of 330 nm and recording TNS emission at 432 nm. Bandwidths of 4 nm for excitation and 4 nm for emission were used. Data were fitted to a single-site binding model.

Determination of Active Antithrombin—The active concentration of full-length antithrombin was determined by stoichiometry of inhibition using 1 μM antithrombin and 20 μM TNS with full-length high-affinity heparin previously standardized against fully active plasma antithrombin, using the well-defined inflection point of the fluorescence at saturation with heparin (15). Measurements were made on an SLM 8000 spectrophotometer, exciting TNS at a wavelength of 330 nm and recording TNS emission at 432 nm. Bandwidths of 4 nm for excitation and 4 nm for emission were used. Data were fitted to a single-site binding model.

Determination of Antithrombin-Heparin Dissociation Constants—Because of the decreased fluorescence enhancement upon heparin binding seen for the antithrombin deletion variants, tryptophan fluorescence, which is normally exploited to determine heparin affinity (15), could not be used to obtain accurate heparin affinities. Instead, the fluorescent probe TNS (Molecular Probes), a probe that is sensitive to the environment and binds weakly to antithrombin (K_D > 125 μM),2 was used. Heparin binding to antithrombin-TNS causes a change in the environment of the antithrombin-bound TNS, resulting in an approximately 50–60% decrease in TNS fluorescence intensity and a blue shift of the emission maximum. To determine the antithrombin-heparin dissociation constants for the variants, antithrombin in the presence of a 20-fold excess of TNS was titrated with heparin. For control antithrombin and the Δ134 variant, 250 nM antithrombin and 5 μM TNS were used for both pentasaccharide and full-length heparin titrations. For the rest of the deletion variants, 250 or 300 nM antithrombin and 5 or 6 μM TNS were used for full-length heparin titrations, and 250–500 nM antithrombin and 5–10 μM TNS were used for pentasaccharide titrations. All titrations were carried out in 1.0 M buffer containing 20 mM sodium phosphate, 0.1 M EDTA, 0.1 M polyethylene glycol 8000, and 1 M NaCl at 25 °C and pH 7.4. Fluorescence measurements were recorded on an SLM 8000 spectrophotometer, exciting the TNS at 330 nm and recording TNS emission at 432 nm. Bandwidths of 4 nm for excitation and 8–16 nm for emission (depending on antithrombin concentration) were used. Data were fitted to a single-site binding model.

Stoichiometry of Inhibition—The stoichiometry of inhibition (SI), defined as the number of moles of inhibitor needed to inhibit 1 mol of proteinase, was measured by incubating antithrombin over a range of concentrations with a constant concentration of proteinase. Reactions were allowed to go to completion, and residual proteinase activity was measured by diluting the reaction mixture into chromogenic substrate.
and measuring the rate of substrate hydrolysis, as described previously (16). The stoichiometry of inhibition was determined by plotting the residual proteinase activity against the ratio of antithrombin to proteinase. For factor Xa, the chromogenic substrate used was 100 μM Spectrozyme Xa (American Diagnostica) and for thrombin 100 μM S2238 (Chromogenix). For factor Xa the stoichiometry of inhibition was measured using 50 nM factor Xa and 10–60 nM antithrombin. For determinations in the presence of heparin, 120 nM full-length heparin was added. For thrombin, the stoichiometry of inhibition was measured using 50 nM thrombin and 10–200 nM antithrombin and, in the presence of heparin, 150 nM HAH. All reactions were carried out in buffer containing 20 mM sodium phosphate, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, and 0.1 mM NaCl (1 0.15) at 25 °C and pH 7.4, in PEG 20,000-coated cuvettes.

**Kinetic Assays**—The rate of inhibition of factor Xa by the antithrombin variants was measured under pseudo-first order conditions of 250 nM antithrombin and 20 nM factor Xa, as described previously (15). For the heparin catalyzed reaction, the second order rate constant $k_{cat}/K_m$ was determined using the above conditions in the presence of catalytic concentrations of full-length heparin (0–4 nM) or pentasaccharide heparin (0–40 nM) by Equation 1 (2).

$$k_{cat} = \frac{[AT]_0}{K_m} + k_{uncat} [AT],$$

(1)

where $[H]_h$ is the total heparin concentration, $[AT]_n$ is the total antithrombin concentration, $K_m$ is the measured antithrombin-heparin dissociation constant, and $k_{uncat}$ is the second order rate constant for inhibition of proteinase in the absence of heparin. The chromogenic substrate used to measure residual factor Xa activity was Spectrozyme Xa (American Diagnostica). Rates of inhibition of thrombin were determined similarly, using 250 nM antithrombin and 10 nM thrombin and, for the heparin catalyzed reaction, 0–1 nM full-length heparin. Residual thrombin activity was measured by the rate of hydrolysis of the chromogenic substrate, S2238 (Chromogenix). All kinetic reactions were carried out in PEG 20,000-coated cuvettes at 25 °C in pH 7.4 buffer containing 20 mM sodium phosphate, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, and 0.1 mM NaCl (1 0.15).

**Tryptophan Fluorescence Emission Spectra**—Tryptophan fluorescence emission spectra were recorded on an SLM 8000 spectrofluorimeter, exciting at 280 nm and recording emission in 1 nm steps from 300–400 nm. An excitation bandwidth of 4 nm and an emission bandwidth of 2 nm were used. The concentration of antithrombin was 500 nM. In the presence of heparin, 1.5 μM full-length heparin was added. For the weakest binding variant, Δ134–137, this gave 93% saturation.

**Materials**—Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Life Technologies, Inc. Human factor Xa was prepared as described previously (17). α-Thrombin was prepared from prothrombin isolated from outdated human plasma by the method of Miletich et al. (18), by reaction with snake venom as described (19). Full-length high affinity heparin (Mv 15,000) was prepared by fractionation of heparin, first by size-exclusion chromatography and then by antithrombin affinity chromatography as described (2). Synthetic high affinity heparin pentasaccharide was a gift from the late Dr. Jean Choay (Center Choay). The plasmids pMAMStop, pRMH140, and pSV2dhfr (13) were gifts from Dr. Gerd Zettlemöll (Behringwerke, Marburg).

**RESULTS**

**Purity and Integrity of Antithrombin Variants**—The five antithrombin deletion variants (Fig. 2) were determined to be homogeneous after purification by analysis on SDS-polyacrylamide gel electrophoresis (data not shown). In the absence of proteinase, all variants migrated as a single band with the same mobility as control antithrombin. Because the mutations represented deletions of one or more residues rather than simple amino acid replacements, it was necessary to show that the changes had not altered the basic properties of the antithrombin molecule. As a serpin, antithrombin inhibits proteinases by forming SDS-stable 1:1 covalent complexes. Because functioning of the serpin mechanism is very sensitive to the conformation of the inhibitor, the ability to inhibit proteinases is a good diagnostic for retention of the basic serpin fold. All of the antithrombin deletion variants were able to form the characteristic higher molecular weight SDS-stable covalent complex with thrombin (not shown). This demonstrated that the deletions in the region linking helix D and strand 2A had not affected the ability to inhibit proteinase by the serpin inhibitory mechanism and implied that the antithrombin variants were correctly folded.

**Stoichiometry of Inhibition**—SI values were determined for the variant antithrombins for reaction with both factor Xa and thrombin. Both in the absence and presence of heparin the deletions caused only modest increase in the SI, with the largest value in the absence of heparin, 1.8–1.9, being found for the Δ134 variant with factor Xa and thrombin (Table I). Compared with control antithrombin, heparin caused similar small increases in SI for the variants. Because the deletions are less likely to have affected the substrate reaction rate constant, it is probable that the small increases in SI for the variants represent small reductions in the rate constant for commitment to the inhibition pathway. This rate constant is influenced by the relative ease of insertion of the reactive center loop into the substrate A at the stage of the acyl enzyme intermediate (20–23). Thus, the small increases in SI for several of the variants may reflect small perturbations of the sheet opening mechanism.

**Fluorescence and Fluorescence Enhancement of the Anti-thrombin Variants**—Human antithrombin contains four tryptophans, at positions 49, 189, 225 and 307. Only tryptophan 49 is directly in the heparin-binding site.Nevertheless, heparin binding results in an ~40% fluorescence enhancement as a result of the heparin-induced conformational changes. We have previously shown that most of the enhancement results from changes in the environment of tryptophan 225 and 307 (24) and is directly linked to expulsion of the P14 residue from β-sheet A and the resulting structural changes arising from sheet contraction (tryptophan 225 is adjacent to P14 in the

**Table I**

| Antithrombin | Factor Xa | Thrombin |
|--------------|-----------|-----------|
|              | -Heparin  | +HAH      | -Heparin  | +HAH      |
| Control      | 1.2 ± 0.1 | 1.7 ± 0.1 | 1.1 ± 0.1 | 1.5 ± 0.1 |
| Δ134         | 1.9 ± 0.7 | 2.7 ± 0.2 | 1.8 ± 0.1 | 2.7 ± 0.5 |
| Δ134–135     | 1.1 ± 0.3 | 1.6 ± 0.04 | 1.0 ± 0.1 | 1.4 ± 0.1 |
| Δ134–136     | 1.9 ± 0.4 | 2.4 ± 0.4 | 1.6 ± 0.1 | 2.3 ± 0.1 |
| Δ134–137     | 1.5 ± 0.1 | 1.5 ± 0.1 | 1.4 ± 0.1 | 2.0 ± 0.2 |
| Δ137         | 1.6 ± 0.1 | 1.5 ± 0.3 | 1.2 ± 0.1 | 1.8 ± 0.1 |

**Table II**

| Antithrombin | Helix D | Helix extension |
|--------------|---------|----------------|
| Control (N135Q) | H F F F A K L N C R L Y R K A Q K - S K | |
native state and tryptophan 307 lies directly below this position on an axis perpendicular to \( \beta \)-sheet A. Changes in the conformation of antithrombin between loop-inserted and loop-expelled conformations are therefore sensitively reported by changes in tryptophan fluorescence.

All variants gave essentially normal emission spectra in the absence of heparin in both intensity and shape when compared with that of control antithrombin (not shown), indicating both that the environments of the tryptophans had not been perturbed by the mutations and also that the ground state conformational equilibrium between loop-inserted and loop-expelled states still very strongly favored the loop-inserted state in each case. The method is not, however, sensitive enough to identify very small percentage changes in the loop-expelled conformation.

The five variants fell into two groups with respect to heparin-induced tryptophan fluorescence changes. The \( \Delta137 \) variant gave an essentially normal enhancement of 35% (Table II). The remaining variants, in which deletions were present near the D helix, showed strongly altered tryptophan fluorescence enhancements, with increases of only 6–15% upon saturation with heparin (\( K_d \) values were determined below). This could be interpreted in one of two ways. If the mutations are affecting the position of equilibrium between A and B states, this would suggest that the percentage of B state induced by heparin (Table II) ranges between 15% for the \( \Delta 134 \) variant and a high of 37% for the \( \Delta 134–135 \) variant, compared with \( \approx 100\% \) normally. Alternatively heparin may in each case induce a new and different conformation in which tryptophans 225 and 307 are perturbed differently. In either case it would suggest that the deletions from the N-terminal end had affected the coupling between the conformational changes induced in the heparin-binding site and the resulting conformational changes in the reactive center loop that are reported by tryptophans 225 and 307.

**Heparin Affinity of Antithrombin Variants and Use of TNS Binding Assay**—Heparin affinity is normally determined by titration of antithrombin with heparin and monitoring changes in tryptophan fluorescence. However, the reduced fluorescence enhancement upon heparin binding for the deletion variants restricted the use of tryptophan fluorescence to monitor the heparin affinity. Instead, TNS, a hydrophobic probe that binds to antithrombin and is sensitive to heparin binding, was employed. The binding of heparin to antithrombin-TNS to the point of saturation of the heparin-binding site resulted in approximately a 60% decrease in TNS fluorescence intensity and a blue shift of about 3 nm (Fig. 3). The saturaability of the TNS fluorescence change with heparin and the altered intensity and wavelength maximum indicate that heparin is not competing with TNS for binding at the same site on antithrombin but rather that TNS is a reporter of the conformation of antithrombin. A control titration of heparin binding to plasma antithrombin showed that the heparin dissociation constants determined in this way agreed with those determined using change in tryptophan fluorescence and so showed that the results were not affected by the presence of TNS (data not shown). Titration of antithrombin-TNS with heparin was therefore used to determine the heparin affinity for all the variants as well as control antithrombin. Although all of the deletion variants showed reduced affinity for both full-length and pentasaccharide heparin (Fig. 4 and Table III), the reductions represented loss of no more than about 18% of the binding energy (2 kcal mol\(^{-1}\) out of 12 kcal mol\(^{-1}\)). For pentasaccharide heparin, deletions near the D helix (\( \Delta134 \)) showed a 10–40-fold reduction in heparin affinity, whereas the deletion further from the D helix (\( \Delta137 \)) showed a smaller reduction (6-fold). For full-length heparin, deletions near the D helix showed a 12–39-fold reduction in affinity, whereas the deletion further away showed only a 5-fold reduction in affinity. To emphasize that these reductions in heparin affinity are relatively modest given the nature of the structural changes, it should be noted that the reactive center loop-cleaved form of antithrombin has lost about 35% of the heparin binding energy relative to native antithrombin, even though residues in the heparin-binding region differ only conformationally from those in the native state.

**Rate of inhibition of Factor Xa**—Based on our prediction of the role of the 134–137 region of antithrombin in mediating the heparin activation of antithrombin, we had expected that one or more of the deletion variants would show a greatly enhanced rate of inhibition of factor Xa in the absence of heparin, perhaps being similar to that of heparin-activated antithrombin. This was found not to be the case. Instead, when corrected for SI, it was found that the basal rates of inhibition of factor Xa were very similar in all cases to that of control antithrombin (Table IV). Indeed there were modest reductions in basal rate of factor Xa inhibition. This implied that, like control antithrombin, all of the variants existed close to 100% in the low activity P14-inserted conformation, perhaps with the loop-inserted conformation even closer to 100% for those. More surprising was the effect that binding heparin had on the rates of inhibition. Whereas the \( \Delta 137 \) variant behaved similarly to control antithrombin and gave

### Table II

| Antithrombin    | Fluorescence enhancement* (%) |
|----------------|-----------------------------|
| Control        | 43                          |
| \( \Delta 134 \)| 14                          |
| \( \Delta 134–135 \)| 15                         |
| \( \Delta 134–136 \)| 6                           |
| \( \Delta 134–137 \)| 9                            |
| \( \Delta 137 \)| 35                          |

* Measured at 340 nm. Experimental errors are ± 10% of observed change.
Thrombin is an N135Q variant that is equivalent to the control antithrombin (Table IV). It should be noted that because the control antithrombin was too tight to accurately determine the affinity of control antithrombin. TNS, a fluorescent probe. Representative titrations of control antithrombin with full-length heparin were determined by heparin titration of antithrombin bound to TNS, a fluorescent probe. Representative titrations of control antithrombin (squares and solid line), Δ134 antithrombin (circles and dashed line), and Δ134–136 antithrombin (diamonds and dot-dashed line) with full-length heparin are shown. The $K_d$ values derived from fits of these titrations, using a single-site binding model, are presented in Table III. Titrations were carried out on 250 nM antithrombin in the presence of 5 μM TNS.

**TABLE III**

| Antithrombin | H5 $K_d$ (μM) | HAH $K_d$ (μM) |
|--------------|---------------|----------------|
| Control      | ~6$^a$        | ~2$^a$         |
| Δ134         | 57 ± 18.0     | 26 ± 4         |
| Δ134–135     | 283 ± 94      | 54 ± 6         |
| Δ134–136     | 220 ± 38      | 58 ± 14        |
| Δ134–137     | 241 ± 20      | 79 ± 15        |
| Δ137         | 37 ± 1.0      | 10 ± 1         |

$^a$ Measurements were done at $I = 0.15$. At this ionic strength binding is too tight to accurately determine the affinity of control antithrombin.

**TABLE IV**

| Antithrombin | −Heparin $k_{obs}$ ($\text{μM}^{-1}\text{s}^{-1}$) | +Heparin $k_{obs}$ ($\text{μM}^{-1}\text{s}^{-1}$) |
|--------------|-----------------------------------------------|-----------------------------------------------|
| Control      | 3.4 ± 0.2                                     | 520 ± 17                                     |
| Δ134         | 1.6 ± 0.4                                     | 85 ± 4                                       |
| Δ134–135     | 2.7 ± 0.1                                     | 21 ± 0.6                                     |
| Δ134–136     | 1.4 ± 0.2                                     | 77 ± 2.4                                     |
| Δ134–137     | 1.6 ± 0.1                                     | 70 ± 6.1                                     |
| Δ137         | 2.6 ± 0.4                                     | 428 ± 15                                     |

**FIG. 4.** Heparin titrations of antithrombin-TNS to determine heparin affinity. The $K_d$ values for full-length and pentasaccharide heparin were determined by heparin titration of antithrombin bound to TNS, a fluorescent probe. Representative titrations of control antithrombin (squares and solid line), Δ134 antithrombin (circles and dashed line), and Δ134–136 antithrombin (diamonds and dot-dashed line) with full-length heparin are shown. The $K_d$ values derived from fits of these titrations, using a single-site binding model, are presented in Table III. Titrations were carried out on 250 nM antithrombin in the presence of 5 μM TNS.

 Rates of Inhibition of Thrombin—In the absence of heparin all of the variants showed rates of reaction with thrombin similar to that of control heparin (Table V). In the presence of full-length heparin all of the variants showed greatly increased rates of inhibition similar to control antithrombin, with the exception of the Δ134–137 variant, which was enhanced to only 10% of the level of the control (Table V). The similar rates for control and four of the five variants is understandable because the heparin-accelerated rate of inhibition of thrombin by antithrombin is less dependent on the conformational state of the reactive center loop and instead depends on a bridging mechanism. Thus, with control antithrombin the rates of inhibition of thrombin by B state, P14-expelled antithrombin is only ~2-fold higher than for the A state P14-inserted conformation (2), and the large rate enhancements observed in the presence of full-length heparin are almost all because of the bridging effect of the heparin. Thus, the apparently normal behavior of most of the variants against thrombin, both in the absence and presence of heparin indicate only that the bridging mechanism has not been significantly compromised by the deletions. This is not the case for the largest deletion, the Δ134–137 variant, which has approximately normal rate of thrombin inhibition in the absence of heparin but shows only 10% of the normal bridging contribution, although without a large effect on the SI (Table I). One possible explanation is that because the deletions occur in what has been predicted to be an extended heparin-binding site, i.e. where residues beyond the necessary pentasaccharide might bind, the deletion of four residues from this region might have altered the extended site interactions and thereby interfered with the template mechanism.

**DISCUSSION**

In this work we examined the role of residues 134–137, which link helix D to β-sheet A, in the heparin activation of antithrombin against factor Xa through transmitting conformational change from the heparin-binding site to the reactive center loop of antithrombin. Based on our original hypothesis that the linkage mechanism is a mechanical pulling on β-sheet A induced by extension of the D helix that might be mimicked...
by the deletions, we had expected that one or more of the deletion variants would show enhanced rate of reaction with factor Xa in the absence of heparin and somewhat increased heparin affinity. Such a finding was previously made for a P14 antithrombin variant, S380W, which had properties consistent with a partial pre-expulsion of the P14 residue in the native state (7). This was not what was found for the present variants. Instead, all of the deletion variants, in which residues were removed from the 134 end, had normal basal rates of inhibition of factor Xa but had lost much of the ability to be activated by heparin toward factor Xa, despite only modest reductions in their heparin affinity. The effects of the sequential deletions on basal rate of factor Xa inhibition, heparin affinity, and the ability of heparin to activate the variants did not, however, result from misfolding of the protein, because neither the conformation-sensitive ability to form SDS-stable complex with proteinase with SI values close to 1, nor the presence of a high affinity heparin-binding site were compromised. Whereas the findings therefore do not support our original hypothesis of the role of residues 134–137 in a mechanical conformational transmission mechanism, they do very strongly implicate these residues in the coupling of conformational changes in the heparin-binding site to the conformational activation of the reactive center loop by some other mechanism.

Consideration of the specific changes in properties of the antithrombin variants that result from the sequential deletions gives some insight into the possible nature of such a mechanism. First, it appears that heparin binding to the Δ134 variants does not cause the same conformational changes as control antithrombin, whether by extent or by type. This is suggested by the reduced changes in tryptophan fluorescence that accompany heparin binding, compared with control antithrombin. Because we have previously established that ~73% of the enhancement in tryptophan fluorescence that normally accompanies heparin binding results from the expulsion of P14 from proximity to tryptophan 225 and from changes in the environment of tryptophan 307 as a result of the subsequent sheet closure (24), we can be reasonably certain that the four deletion variants that include deletion of residue 134 are unable in large part to bring about full loop expulsion. Depending on whether the variants have only A and B state conformations available to them versus having unique activated conformations, C, D, etc., this could be interpreted in one of two ways. If there is an equilibrium between only A and B states, we would expect that the fractional fluorescence enhancement would correlate directly with the fractional heparin-induced rate enhancement for inhibition for factor Xa. We found, however, that this is not the case, with instead poor agreement between the rate enhancement predicted from fluorescence enhancement and that determined experimentally. This agreement becomes even poorer if part of the observed fluorescence enhancement results from the fraction of the normal enhancement that arises from tryptophans 49 and 189 (100–73 = 27%). Alternatively, heparin binding may fully convert each variant to a unique conformation, which differs in each case from the normal activated B state with respect to the changes involving β-sheet A and the reactive center loop and hence in both the observed fluorescence enhancement and the increase in rate of inhibition of factor Xa. Although such an explanation seems less pleasing from the point of view of maintaining the simplest model of heparin activation of antithrombin, it does seem to be more compatible with the data presented, given the irregular relationship between fluorescence enhancement and rate of factor Xa enhancement caused by heparin.

At first sight the reduced fluorescence enhancements and smaller increases in rate of inhibition of factor Xa may appear to parallel the behavior of low affinity heparin (LAH) binding to antithrombin. Here, at saturation, there is a much smaller increase in tryptophan fluorescence than is found with HAH (~16% versus 40%) and a reduced enhancement of factor Xa inhibition (~60% of maximum) (3, 4). However, this situation differs from that of the present variants in that heparin affinity is much reduced for LAH compared with HAH, whereas heparin affinity for the deletion variants is only modestly reduced compared with control. It has been well documented that heparin binding occurs in two steps, a first low affinity interaction (~20 μM $K_D$) (2) followed by a conformational change step that (i) represents the conformational activation as the A-B equilibrium shifts toward B and (ii) gives a conformation in the heparin-binding site that binds the specific heparin pentasaccharide much more tightly. The result is that for HAH the B/A ratio is ~1000:1. Low affinity heparin binds much less tightly in the B state than does HAH, and the B/A ratio is ~1. This is reflected in the very much lower affinity of LAH versus HAH. For the deletion variants, however, the reduction in heparin affinity is much less than between LAH and HAH (maximally 40-fold) so that, if there were still a simple A to B equilibrium, there should be enough energy of heparin binding in the second step to ensure a B/A ratio >1. Indeed, a heparin trisaccharide that binds to antithrombin 100-fold less tightly than pentasaccharide still gives B/A of 8 and consequently close to normal enhancement of factor Xa inhibition and of tryptophan fluorescence (25). Even if the effect of the deletions were, unexpectedly, to cause an increase in stability of the loop-inserted form, so that an increased part of the heparin binding energy would be needed for loop expulsion, there should still be sufficient heparin binding energy available to ensure that close to a complete shift in conformational equilibrium could be effected by heparin binding, based on the following calculations. Using the somewhat reduced basal rates of factor Xa inhibition of the variants compared with control antithrombin as an indication of a small shift in the inserted-expelled equilibrium and using a rate constant of $5 \times 10^5$ M$^{-1}$ s$^{-1}$ for ~100% loop-expelled conformation, one can estimate that the control antithrombin in the absence of heparin (basal rate of Xa inhibition of $3.4 \times 10^3$ M$^{-1}$ s$^{-1}$; Table IV) is approximately 0.7% expelled and 99.3% inserted, assuming negligible activity for the loop-inserted conformation. A reduction of basal rate to $1.6 \times 10^3$ M$^{-1}$ s$^{-1}$ (Δ134 variant) might thus result from a reduction in the expelled conformation to 0.3%. If this were to arise from an increased stability of the loop-inserted form it would correspond to a stabilization of $-RT \ln (0.7/0.3) = 0.5$ kcal mol$^{-1}$. It has been shown for plasma antithrombin, however, that the conformational change component of heparin binding corresponds to a net favorable binding energy contribution of $RT \ln 1000 = 4.1$ kcal mol$^{-1}$ (2). With an increased stability for the inserted state of 0.5 kcal mol$^{-1}$, this favorable contribution would be reduced to 3.6 kcal mol$^{-1}$. This still corresponds to an equilibrium of 99.7% expelled in the heparin-bound state. Even if, in addition, the full reductions seen in heparin affinity for the variants resulted from reductions in the conformational change step (6–47-fold; Table III), there would still be sufficient binding energy left for this step (3.6 minus 2.2 kcal mol$^{-1}$) to ensure ~90% in the loop-expelled state, even for the variant with the largest reduction in heparin affinity (Δ134–135). For the Δ134 variant, the ~10-fold reduction in affinity for pentasaccharide (Table III) would leave a minimum of 2.1 kcal mol$^{-1}$ from conformational change/loop expulsion and 97% in the loop expelled form upon heparin binding. We must therefore conclude for the deletion variants that the failure to obtain maximal enhancements of tryptophan fluorescence and of rates of inhibition of factor Xa upon heparin binding, as would be
expected from a B/A ratio >1, again suggests that the coupling between the heparin-binding site and the reactive center loop has been disrupted and that the effect of heparin binding in each case is to give rise to unique conformations for the reactive center loop, each of which has a different rate of reaction with factor Xa than does either the A or B state of control antithrombin.

The question is therefore what the nature of such disruption might be. Here it is significant that, whereas removal of residue 134 is sufficient to bring about much of the perturbation in properties seen with the longer deletions, removal of residue 137 has little effect. This demonstrates that the partial decoupling of heparin binding from conformational activation either specifically requires Ala134 or else the conformation of residues beyond this that depend on Ala134 being present. Here it may be significant that the normal extension of the D helix that is induced by heparin binding involves a stretch of six residues that include three basic residues, Arg132, Lys133, and Lys136. The separation of three residues between the two lysines enables them both to be on the outer face of the elongated helix, exposed to solvent. However, upon removal of one or both of the intervening residues, this would no longer be the case and might compromise the tendency of the preceding residues to form an extension to the D helix. Resolution of this question will require crystallization of one or more of the deletion variants.

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