Antithrombin (AT) inhibits most of the serine proteases generated in the blood coagulation cascade, but its principal targets are factors IXa, Xa, and thrombin. Heparin binding to AT, via a specific pentasaccharide sequence, alters the conformation of AT in a way that promotes efficient inhibition of factors IXa and Xa, but not of thrombin. The conformational change most likely to be relevant to protease recognition is the expulsion of the N-terminal portion of the reactive center loop (hinge region) from the main β-sheet A. Here we investigate the hypothesis that the exosites on the surface of AT are accessible for interaction with a protease only when the hinge region is fully extended, as seen in the related Michaelis complex between heparin cofactor II and thrombin. We engineered a disulfide bond between residues 222 on strand 3A and 381 in the reactive center loop to prevent the extension of the hinge region upon pentasaccharide binding. The disulfide bond did not significantly alter the ability of the variant to bind to heparin or to inhibit thrombin. Although the basal rate of factor Xa inhibition was not affected, that of factor IXa inhibition was reduced to the limit of detection. In addition, the disulfide bond completely abrogated the pentasaccharide accelerated inhibition of factors Xa and IXa. We conclude that AT hinge region extension is the activating conformational change for inhibition of factors IXa and Xa, and propose models for the progressive and activated AT Michaelis complexes with thrombin, factor Xa, and factor IXa.

The serpin antithrombin (AT) is capable of inhibiting most of the serine proteases generated in the blood coagulation cascade. Its central role is illustrated by the embryonic lethal phenotype of the AT knock-out mouse (1) and by the success of therapeutic heparin. Heparin exerts its anticoagulant effect primarily through an interaction with AT, due to the presence of a specific pentasaccharide sequence found in one-third of heparin chains (2, 3). The binding of the isolated pentasaccharide to AT catalyzes the inhibition of factors IXa and Xa by ~300-fold but does not appreciably affect the rate of thrombin inhibition (4, 5). Thrombin inhibition by AT is accelerated by approximately four orders of magnitude (6) in the presence of heparin chains of at least 18 residues in length (7), due to the obligate co-occupation of thrombin on the same heparin chain. The heparin activation mechanism of AT toward factors IXa and Xa is thus allosteric, but it is not clear which of the heparin-induced conformational changes in AT is responsible for the improvement in rate of inhibition.

The AT conformational changes that take place in response to heparin binding are well characterized (8–11) and are summarized in the first two panels of Fig. 1. They include the N- and C-terminal elongation of helix D in the heparin binding region and the expulsion of the N-terminal portion (hinge region) of the reactive center loop (RCL) from β-sheet A. Tertiary structural changes also occur in response to heparin binding, the most significant of which is the rotation of the upper β-barrel domain on the lower helical domain resulting in an alternation of the surface properties of AT. It has been proposed that pentasaccharide-accelerated inhibition of factors IXa and Xa is the result of the stabilization of the initial recognition (Michaelis) complex through the exposure of exosites on the surface of AT (12–14). This hypothesis is strengthened by the insensitivity of the catalytic effect of the pentasaccharide to the sequence of the RCL (15) and by the effect of mutations on the surface of AT (16).

We have modeled the Michaelis complexes of AT with factor Xa and thrombin based on the crystallographic structure of the related serpin heparin cofactor II (HCII) in its Michaelis complex with thrombin (17, 18). Our hypothesis is that the exosite for factor IXa and Xa docking on AT is similar to that found for thrombin docked on HCII. The predicted Michaelis complex is given as the third panel in Fig. 1 and suggests an allosteric activation mechanism dependent on the expulsion of the hinge region from β-sheet A and its full extension toward the top of AT. According to the model, the low basal rate of AT inhibition of factors IXa and Xa would be due to the restricted flexibility of the hinge region of the RCL, which would effectively prevent the simultaneous engagement of the exosite and the reactive center by the protease. Our proposed model for the AT-thrombin Michaelis complex, on the other hand, would not require the extension of the hinge region and is consistent with native AT inhibiting thrombin as well as allosterically activated AT. One implication of our hypothesis is that the uncatalyzed, or progressive, Michaelis complex between AT and its targets will preserve the exosite interactions formed in the presence of heparin. The appreciable basal rates of AT inhibition of factors IXa and Xa would be due either to a rapid equilibrium resulting in the presence of a small fraction of the activated conformation of AT in the absence of the pentasaccharide (19, 20), or to an induced fit mechanism whereby the initial weak interaction between AT and protease could extract the hinge region from β-sheet A to allow the engagement of the exosite. The effect of...
the pentasaccharide on the observed rates of protease inhibition would be due to a repositioning of the equilibrium in favor of the hinge region expelled conformation.

We have directly tested the hypothesis that hinge region extension is critical for the formation of the AT-protease Michaelis complexes by engineering a disulfide bond between the hinge region (Glu-381–Cys) and strand 3 (Lys-222–Cys) of β-sheet A (Fig. 2). Although this disulfide bond does not prevent hinge region expulsion, it does effectively prevent its extension. Formation of the new disulfide bond was confirmed by SDS-PAGE and by DTDP assay. The variant bound the heparin pentasaccharide with near wild-type affinity and inhibited factors IXa, Xa, and thrombin with only slightly elevated stoichiometries. As predicted, the rate of thrombin inhibition was insensitive to the presence of the new disulfide bond, both in the absence and presence of heparin. Inhibition of factor Xa was not affected by the disulfide bond in the absence of the pentasaccharide, but the variant was no longer activated toward factor Xa in the presence of the pentasaccharide. The ability of the AT variant to inhibit factor IXa was reduced to the limit of detection, and inhibition was not accelerated by the pentasaccharide. We conclude that the conformational change responsible for the allosteric activation of AT is the extension of the hinge region from β-sheet A and that the Michaelis complexes of pentasaccharide-activated AT with factors IXa and Xa resemble that found in the crystal structure of HCII with thrombin.

EXPERIMENTAL PROCEDURES

Materials—Human α thrombin and factor IXa were obtained from Hematologic Technologies (Essex Junction, VT), and human factor Xa was from Enzyme Research Laboratories (Swansea, UK). The heparin pentasaccharide (fondaparinux) was kindly provided by Maurice Petitou (Sanofi-Synthelabo, Toulouse, France), and full-length unfractionated heparin (FLH) with an average molecular mass of 15 kDa was purchased from Rovi Pharmaceutical Laboratories (Madrid, Spain). The active concentrations of the pentasaccharide and FLH were determined by stoichiometric titration into a known concentration of plasma AT.

AT Expression and Purification—The K222C and E381C single and double mutations were constructed on the recombinant β-glycoform S137A AT background to reduce glycosylation heterogeneity and facilitate purification though improved heparin affinity (21). S137A is sometimes referred to in the text as “control,” and the S137A/K222C, S137A/E381C, and S137A/K222C/E381C variants are referred to as K222C, E381C, and K222C/E381C (or the “double mutant”) for clarity. The human AT variants were created by site-directed mutagenesis of the pMA-AT plasmid using the Stratagene QuikChange site-directed mu-
tagenesis kit using the appropriate primers for the mutations described. The pMA-AT expression plasmids were stably cotransfected into competent baby hamster kidney cells with pRMI140 and pSV2HSV plasmids using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) and selected by resistance to neomycin and methotrexate (Sigma) as previously described (22). Cells were grown to confluence in triple flasks, and AT was purified from serum-free media. Purification was achieved by a combination of affinity chromatography on heparin-Sepharose and anion exchange chromatography using Q-Sepharose. Heparin-Sepharose chromatography yielded two active peaks, a low and high affinity form, eluting at around 1.0 and 1.5 M NaCl, respectively, as described previously for N135Q AT (23). All experiments were carried out on the high affinity (non-fucosylated) form. Purified material was stored at −80 °C prior to use. Concentration of AT was determined by absorbance at 280 nm and was judged to contain no significant fraction of unreactive material (e.g., latent or polymeric) by its ability to fully react with thrombin by SDS-PAGE (Fig. 3B). Complete formation of the novel disulfide bond between Cys-222 and Cys-381 was confirmed by non-reducing SDS-PAGE (Fig. 3A) and by a 4,4′-dithiodipyridine (DTDP) assay, as done before (24) (data not shown).

Stoichiometries of Inhibition—Stoichiometries of AT inhibition of factors IXa, Xa, and thrombin were determined in the presence and absence of equimolar concentrations of pentasaccharide, and stoichiometries of thrombin inhibition were also determined in the presence of equimolar FLH. For factor Xa and thrombin, the protease was incubated at room temperature with increasing concentrations of AT in 50 mM Tris, 100 mM NaCl, 0.1% bovine serum albumin, pH 7.4 (TBSA), and factor IXa was incubated in 100 mM Hepes, 100 mM NaCl, 0.1% polyethylene glycol, 5 mM CaCl₂, 0.1% BSA, pH 7.4 (HBSA) (35). Each reaction (1/4 of final volume) was allowed to go to completion, and due to the differences in stoichiometries and rates, the reaction conditions and incubation times depended on the AT variant/protease pair. For all of the control AT variants (S137A, K222C, and E381C) with factor Xa and thrombin the protease concentration was 100 nM and AT concentrations ranged from 0 to 600 nM, and incubation times were between 2 and 16 h. For the double mutant (K222C/E381C) with factor Xa and thrombin the protease concentration was 500 nM and the AT concentration ranged from 0 to 2500 nM, with incubation times between 24 and 72 h. The slow rate of factor IXa inhibition required higher concentrations and longer incubation times. For the controls, the factor IXa concentration was between 500 and 2000 nM, incubation times were between 44 and 72 h; for the double mutant the factor IXa concentration was between 2000 and 4000 nM, and incubation times were between 18 and 2 days. Long incubation periods required for the reaction of the double mutant with factor IXa raised concerns about the cause of the loss of protease activity. For all experiments a control incubation containing no AT was included, and chromogenic activity was tested before and after the incubation periods. In all cases, the protease was stable for the duration of the incubation, so any reduction in chromogenic activity was due to inhibition of factor IXa by AT. Complex breakdown is also a potential issue with the long incubations required to determine the stoichiometries of factor IXa inhibition by the double mutant, but as the stoichiometries approached lower values with longer incubations, complex breakdown was judged not to artificially inflate the apparent stoichiometries. All reactions were quenched by the addition of 200 μl of chromogenic substrate, and residual protease activity determined by monitoring color generation at 405 nm on a Thermomax plate reader (Molecular Devices, Winnersh, UK). 300 μl of protease (final concentration, 250 nM) was incubated between 2 min and 8 h, in HBSA at room temperature with an equal volume of AT to an inhibitory concentration of wild-type AT was subjected to energy minimization the hinge extended toward the top of AT and the 374–383 distance increased to 23 Å. D, for the disulfide-bonded variant, the same energy minimization regime resulted in movement of the RCL to the right, but extension toward the top of AT was prevented.

Absence of the pentasaccharide were determined essentially as reported previously (5). For the S137A, K222C, and E381C variants, 10 μl of IXa (final concentration, 250 nM) was incubated between 2 min and 8 h, in HBSSA at room temperature with an equal volume of AT to an inhibitory concentration 10-fold that of the protease. For the K222C/E381C variant 1 μl factor IXa was used, incubation periods were extended up to 54 h, and due to some uncertainty over the exact SI, the concentration of the AT variant was 50-fold that of factor IXa. Observed pseudo first order rate constants (k_{obs}) were taken as the slope of the linear plot of the natural log of residual protease activity versus time. The second order rate constants (k_{app}) were taken as k_{obs} divided by the total AT concentration. Pentasaccharide- or FLH-catalyzed second order rate constants were derived by determining k_{obs} at a range of heparin concentrations from 5 to 80 nM for the pentasaccharide, or 0.5 to 4 nM for FLH. The slope of the linear plot of k_{obs} versus heparin concentration gave values for the heparin-accelerated k_{app} (because the K_{a} of the heparin-AT interaction was significantly lower than the total AT concentration (25)). When little or no pentasaccharide activation was observed, the value for the accelerated k_{app} was determined in the presence of saturating pentasaccharide.

Fluorescence Studies—Equilibrium dissociation constants (K_{a}) for the AT-pentasaccharide interaction were determined essentially as described previously (26). Briefly, change in intrinsic fluorescence of AT (25–50 nM) upon titration of the pentasaccharide was monitored at 340
nm on a PerkinElmer Life Sciences 50B spectrofluorometer, with excitation at 280 nm and using bandwidths of 3.5 nm for both excitation and emission. All titrations were carried out at room temperature under physiological ionic strength (I = 0.15) in 20 mM NaPO₄, 100 mM NaCl, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4. Fluorescence emission intensity was taken as the average of 100 measurements recorded at 1-s intervals for each addition of pentasaccharide. Data were fitted as previously described (27). Fluorescence spectra were taken under similar conditions, with the AT concentration at 1 μM and slits reduced to 2.5 nm. To determine the spectral changes associated with pentasaccharide binding, the pentasaccharide was added to a final concentration of 5 μM. Each spectrum was the average of five scans taken using the same sample.

RESULTS

Design and Confirmation of Disulfide Bond—To test the hypothesis that extension of the hinge region is essential for the allosteric activation of AT, we designed a disulfide bond between hinge region residue 381 and strand 3A residue 222 (Fig. 2). These residues were targeted for mutation to cysteines, because they provided the correct distance and geometry for disulfide bond formation, and because the new disulfide bond would restrict the extension of the hinge region when expelled by pentasaccharide binding. In the crystal structures of native and pentasaccharide-bound AT the hinge regions are in similar positions due to the constraints imposed upon the RCL by an interaction with the latent counterpart of the active/latent heterodimer (8, 10, 11). The top of AT and the hinge region forms an S-shape, and the degree of extension of the hinge region can be quantified by the distance between the two ends of the S, defined by Glu-374 (P20) and Ala-383 (P11) in AT (substrate numbering of Schechter and Berger (28)). The distance between these residues is 10.0 Å in the structure of native AT (Fig. 2A) and 16.4 Å in the structure of pentasaccharide-bound AT. In native HCII, which has a similar hinge region conformation as native AT, the distance between equivalent residues (Thr-425 and Thr-434) is 10.7 Å, whereas in its Michaelis complex with thrombin the hinge adopts an extended conformation with a P20–P11 distance of 22.5 Å. Thus, nearly all of the flexibility required for the observed 17.1-Å movement of the HCII P1 residue in making the Michaelis complex with thrombin is provided by the extension of the hinge region. To test the effect of an engineered disulfide bond between residues 222 and 381 on the ability of the hinge region of AT to extend, we created a model of the double variant on the template of pentasaccharide-activated AT and energy-minimized the model for 5000 cycles using the program CNS (29). We found that the presence of the disulfide bond between 222 and 381 limits the possible P20–P11 separation to a maximal distance of 16.8 Å (Fig. 2D). Although this distance is somewhat greater than that found for native AT, the movement of P11 is toward the side of AT, not toward the top as is seen in HCII. In the context of wild-type AT, the same minimization regime resulted in a 23.2-Å separation between P20 and P11, with the P11 displacement straight back toward the top of AT (Fig. 2C).

The presence of the engineered disulfide bond was verified in a number of ways. First, the increased mobility of the variant on a non-reducing SDS-gel indicates a more compact structure when denatured by SDS, consistent with an extra disulfide bond (Fig. 3A). There is no evidence of a wild-type, lower mobility band that would correspond to incomplete formation of the 222–381 disulfide bond. In addition, no high molecular weight polymeric species were observed, as would be expected if a significant fraction of the molecules possessed free, solvent-exposed cysteines. Under reducing conditions the mobility of all AT variants was identical (Fig. 3A). The oxidized state of the additional cysteines at 222 and 381 was also confirmed by DTT assay. Although the single mutants K222C and E381C were able to react with DTT, the control S137A and the double mutant K222C/E381C were unreactive (data not shown). Thus, the designed disulfide bond is correctly formed in the K222C/E381C mutant, and although sterically capable of
hinge region *expulsion* from β-sheet A, modeling studies confirm that it is not capable of hinge region *extension*.

**Heparin Binding Studies**—Although the disulfide bond between strand 3A and the hinge region was not predicted to prevent loop expulsion, we did expect it to alter the heparin-binding properties of the double mutant. Such an expectation was based on the mechanism of heparin binding outlined in Fig. 1. AT binds the pentasaccharide via an induced-fit mechanism where the conformational change step locks AT in a high affinity conformation (30). A 40% enhancement of intrinsic AT fluorescence is the signal commonly used to follow the AT conformational change, and it has been shown to derive from the expulsion of the hinge region and the consequent closing of β-sheet A to the five-stranded form (31). The presence of a disulfide bond between the hinge region and the adjacent strand on β-sheet A would predictably favor the native conformation, resulting in a reduction in heparin affinity. The heparin affinity of the K222C/E381C variant, however, was not altered by the presence of the new disulfide bond. This was initially observed during purification when the double mutant eluted from the heparin-Sepharose column at the same NaCl concentration as the controls (data not shown), and was confirmed by determining the equilibrium dissociation constants of the AT variants for the pentasaccharide by following change in intrinsic fluorescence (Fig. 4). The dissociation constants ($K_d$ values) were $3.3 \pm 0.3$, $11.8 \pm 0.2$, $12.2 \pm 0.1$, and $12.1 \pm 0.4$ nM for AT variants S137A, K222C, E381C, and the disulfide bonded variant K222C/E381C, respectively. The similarity in $K_d$ values obtained for the two single variants and the double variant is remarkable. The same 4-fold reduction in binding affinity was previously observed for the E381A variant, which was explained by a stabilization of the native conformation (26). Clearly the K222C variant could not have the same effect on the native fold of AT as the E381A variant and is more likely to destabilize the activated state, but why the double mutant does not, at least, produce an additive effect (expected $K_d$ of 46 nM) is unclear. Nor is it clear why the disulfide bond does not result in a dramatic reduction in affinity for the pentasaccharide, however, recent crystallographic studies indicate that it may not be necessary to expel the hinge region to obtain the high affinity complex with the pentasaccharide (32, 33).

Although the pentasaccharide affinities of the cysteine variants do not differ greatly from that found for the control S137A, the maximal fluorescence enhancements associated with the interaction do. The titrations shown in Fig. 4 demonstrate an additive effect of the K222C and the E381C mutations on the maximal fluorescence enhancement observed for the disulfide-bonded variant. More accurate values for the fractional fluorescence enhancement were obtained from the spectra of the AT variants before and after addition of saturating pentasaccharide (data not shown), yielding 0.43, 0.20, 0.27, and 0.12 for S137A control, K222C, E381C, and K222C/E381C, respectively. Because the fractional fluorescence enhancement is a measure of the equilibrium position between a fluorescently silent pentasaccharide-bound conformation (presumably hinge region-inserted) and a pentasaccharide-bound state with increased quantum yield (presumably with a five-stranded β-sheet A), it is possible to calculate free energy values relative to the control for comparison (assuming the fractional fluorescence change for S137A corresponds to full conversion to the 5-stranded form). Thus, ∆ΔG values of 0.46, 0.27, and 0.75 kcal/mol were obtained for the K222C, E381C, and K222C/E381C variants, respectively. The effect of the two mutations on the maximal fluorescence enhancement of the double mutant is perfectly additive (0.72 versus 0.75 kcal/mol) and is thus due to the individual point mutations, not to the disulfide bond. This is a perplexing observation, but it is consistent with the finding that the $K_d$ for the pentasaccharide is not affected by the disulfide bond and that the difference in pentasaccharide

![Fractional fluorescence change](image)

**FIG. 4. Pentasaccharide binding titrations reveal normal binding affinity, but reduced fluorescence enhancement.** The intrinsic fluorescence change upon pentasaccharide addition was followed for AT variants S137A (circles), E381C (triangles), K222C (inverted triangles), and K222C/E381C (diamonds). Although the dissociation constants obtained from the fits (solid lines) were similar for all variants, the maximal fluorescence change was significantly reduced for the cysteine variants. The reduction in maximal fluorescence change is additive for the K222C/E381C variant and is thus not due to the formation of the disulfide bond.

| Antithrombin | H5 | FLH | Thrombin | Factor Xa | Factor IXa |
|--------------|----|-----|----------|-----------|-----------|
|              |     |     | SI $k_{app}$ | $k_{app} \times SI$ | SI $k_{app}$ | $k_{app} \times SI$ | SI $k_{app}$ | $k_{app} \times SI$ |
| S137A       | –   | –   | 0.97 ± 0.02 | 8.9 ± 0.2 × 10⁴ | 7.8 ± 10⁴ | 0.94 ± 0.01 | 5.8 ± 0.1 × 10⁵ | 5.5 ± 10⁵ | 1.13 ± 0.08 | 85 ± 1 | 96 |
| +           | –   | –   | 1.07 ± 0.01 | 1.2 ± 0.1 × 10⁴ | 1.3 ± 10⁴ | 1.00 ± 0.05 | 9.3 ± 0.8 × 10⁵ | 9.3 ± 10⁵ | 1.22 ± 0.23 | 3.2 ± 0.5 × 10⁴ | 3.9 ± 10⁴ |
| K222C       | –   | –   | 1.12 ± 0.02 | 1.3 ± 0.1 × 10⁴ | 1.5 ± 10⁴ | ND | ND | ND | ND | ND | ND |
| +           | –   | –   | 1.06 ± 0.06 | 7.1 ± 0.1 × 10⁴ | 7.5 ± 10⁴ | 1.03 ± 0.08 | 2.9 ± 0.1 × 10⁵ | 3.0 ± 10⁵ | 1.22 ± 0.11 | 35 ± 8 | 43 |
| E381C       | –   | –   | 1.11 ± 0.08 | 9.0 ± 0.6 × 10⁵ | 1.0 × 10⁶ | 1.01 ± 0.06 | 3.7 ± 0.1 × 10⁶ | 3.7 ± 10⁶ | 1.27 ± 0.26 | 1.3 ± 0.1 × 10⁷ | 1.7 ± 10⁷ |
| +           | –   | –   | 1.35 ± 0.05 | 11.1 ± 0.1 × 10⁵ | 1.5 × 10⁶ | ND | ND | ND | ND | ND | ND |
| K222C/E381C | –   | –   | 1.15 ± 0.10 | 4.4 ± 0.1 × 10⁶ | 5.1 × 10⁶ | 1.31 ± 0.10 | 1.2 ± 0.1 × 10⁶ | 1.6 × 10⁶ | 1.33 ± 0.08 | 9.3 ± 0.6 | 12 |
| +           | –   | –   | 1.20 ± 0.01 | 6.7 ± 0.5 × 10⁶ | 8.0 × 10⁶ | 1.33 ± 0.01 | 2.7 ± 0.3 × 10⁶ | 3.6 × 10⁶ | 1.33 ± 0.01 | 7.9 ± 1.5 × 10⁵ | 11 × 10⁴ |
|              | –   | –   | 1.43 ± 0.02 | 3.8 ± 0.5 × 10⁶ | 5.4 × 10⁶ | ND | ND | ND | ND | ND | ND |
| K222C/E381C | –   | –   | 2.17 ± 0.07 | 2.7 ± 0.1 × 10⁷ | 5.9 × 10⁷ | 4.27 ± 0.01 | 2.1 ± 0.1 × 10⁷ | 9.0 × 10⁷ | <2.5 | 0.15 ± 0.01 | <0.4 |
| +           | –   | –   | 2.14 ± 0.10 | 3.9 ± 0.1 × 10⁷ | 8.3 × 10⁷ | 3.84 ± 0.01 | 2.4 ± 0.2 × 10⁷ | 9.0 × 10⁷ | <2.5 | 0.20 ± 0.01 | <0.5 |
|              | –   | –   | 5.48 ± 0.10 | 4.8 ± 0.1 × 10⁸ | 2.6 × 10⁸ | ND | ND | ND | ND | ND | ND |

*Values obtained with saturating pentasaccharide.

*ND, not determined.*
binding energy between the control S137A and the disulfide bonded mutant is 0.8 kcal/mol.

Stoichiometry of Protease Inhibition—It was not clear when we designed the double mutant if the new disulfide bond between residues 222 and 381 would hinder the rapid incorporation of the RCL required of the protease inhibition mechanism employed by the serpins (34). To visualize the inhibitory activity of the AT variants we ran a reducing SDS-gel of purified samples, before and after reaction with an excess of thrombin in the presence of catalytic FLH (Fig. 3B). We found that all of the variants, including the double mutant, were capable of thrombin inhibition, as evident from the higher molecular mass band formed upon reaction with thrombin. Some cleaved AT was seen for all AT variants tested indicating slightly elevated stoichiometries of inhibition (SI), especially for the double mutant. Accurate SI values were determined for all AT variants for factors IXa and Xa in the absence and presence of the pentasaccharide, and for thrombin in the absence and presence of both the pentasaccharide and FLH, and the results are given in Table I. It is immediately clear that the effect of the double mutation on the SI values is not additive and that the stoichiometries depend on the protease and whether heparin was used. This provides further confirmation of the correct formation of the engineered disulfide bond, because a small fraction of reduced K222C/E381C would yield the same SI for each protease, and would differ from the single cysteine mutants in an additive manner.

Kinetics of Protease Inhibition—The goal of this study was to determine the effect of a hinge region-restricting disulfide bond on the rate of AT inhibition of its target proteases, in the absence and presence of activating heparin. This was only possible if the heparin-binding mechanism and the protease inhibitory machinery were not significantly perturbed by the new disulfide bond. We have established in the previous sections that the disulfide bond is formed and that it has a relatively minor effect on the ability of AT to bind heparin and to inhibit proteases. Thus, progressive (no heparin) and heparin-activated inhibitory rate constants were determined for the AT variants with thrombin, factor Xa, and factor IXa, and the results are given in Table I.

It has been known for many years now that thrombin inhibition by AT is insensitive to the pentasaccharide-induced AT conformational change. Because we have structural and biochemical data that expulsion of the hinge region from β-sheet A is a part of the activating conformational change, we predicted that restriction of the hinge region by the disulfide bond between 222 and 381 would not affect the rate of thrombin inhibition. This is indeed what we found (Table I). The progressive rate constant for thrombin inhibition is not significantly affected by any of the mutations, nor is it significantly improved by the addition of saturating pentasaccharide. The disulfide bond also has no effect on the ability of FLH to accelerate the inhibition of thrombin (Fig. 5A), when stoichiometry of inhibition is accounted for. Thus, we can conclude that hinge region extension is not necessary to form the Michaelis complex between AT and thrombin, in the absence or presence of activating heparin.

In contrast, the rate of factor Xa inhibition is known to be sensitive to the conformational state of AT. Accordingly, we observed a reduction in the progressive rate of factor Xa inhibition for our single and double mutants (Table I). However, when the change in rate constants relative to the control S137A were calculated in energetic terms, the effect of the disulfide bond was found to be fully additive. The ΔG values for the single mutants K222C and E381C were 0.36 and 0.72 kcal/mol, respectively, yielding an additive ΔG of 1.08 kcal/mol, which is nearly identical to that obtained for the double mutant, 1.06 kcal/mol. However, in the presence of the pentasaccharide, the single mutants each inhibited factor Xa with the expected two orders-of-magnitude increase in rate constant, while inhibition

**Fig. 5. The effect of heparin on observed rates of protease inhibition by AT.** When the rates of protease inhibition are too fast to measure accurately at saturating heparin concentrations, rate constants are obtained at several low heparin concentrations, and the maximal catalysed rate is calculated from the slope of the linear plot of observed rate constant versus heparin concentration. **A,** the rate of thrombin inhibition by all AT variants (symbol styles as in Fig. 4) increases linearly with the addition of FLH. Although the slopes are not identical due to different AT concentrations and SI values, when SI is accounted for the cysteine variants inhibit thrombin as well as the control. **B,** however, while the cysteine variants do not inhibit factor Xa as well as the control, the rates are still responsive to pentasaccharide addition. In contrast, the rate of factor Xa inhibition by the double variant K222C/E381C was entirely insensitive to the pentasaccharide, and even at saturating pentasaccharide no acceleration was observed. **C,** as for factor Xa, factor IXa inhibition by the K222C/E381C variant was wholly insensitive to the pentasaccharide, whereas inhibition by the individual cysteine variants was still accelerated.
Antithrombin Hinge Region Extension

by the double mutant was not accelerated. The rate of factor Xa inhibition by the K222C/E381C AT variant in the presence of the pentasaccharide was indistinguishable from that obtained in its absence, even when a saturating concentration of the pentasaccharide (2 \(\mu\)M) was used (Fig. 5B). This is direct evidence that two distinct Michaelis complexes can be formed between AT and factor Xa: a progressive Michaelis complex formed by the docking of factor Xa on the native conformation of AT; and the activated Michaelis complex requiring the extension of the hinge region of AT, as seen in the Michaelis complex between HCII and thrombin (17). In addition, this finding independently confirms the quantitative formation of the engineered disulfide bond.

Activation of AT inhibition of factor IXa is believed to mirror that of factor Xa inhibition, with a similar acceleration in rate provided by pentasaccharide binding (5). Each single cysteine mutation resulted in a reduction in progressive rate constant of factor IXa inhibition which paralleled that observed for factor Xa. The rate constant for the K222C variant is 2-fold smaller than the control S137A AT, for both factor Xa and IXa, and the E381C variant is 5- and 8-fold slower for factors Xa and IXa, respectively. However, although the disulfide bond has no effect on the progressive rate of factor Xa inhibition, it effectively reduced the rate of factor IXa inhibition to the limit of detection. Due to the extremely slow rates, accurate values for the SI could not be determined; instead, upper limits were determined that approached the value of one as incubation time was increased. We did, however, determine an accurate rate constant for factor IXa inhibition by the double mutant, and when multiplied by the SI value of 2.5, obtained from the longest incubation, an upper limit of 0.4 M\(^{-1}\)s\(^{-1}\) was calculated. This value is not consistent with an additive effect of the individual cysteine mutations and reflects the effect of the disulfide bond between residues 222 and 381. Addition of the pentasaccharide resulted in the expected several hundred-fold increase in the rate of factor IXa inhibition by the control and the single cysteine mutants, but had no effect on the double mutant, even when pentasaccharide concentration was saturating (50 \(\mu\)M) (Fig. 5C). Thus, in contrast to factor Xa, inhibition of factor IXa by AT requires the formation of a single Michaelis complex, both in the absence and presence of the heparin pentasaccharide, in which the hinge region of AT is in an extended conformation.

DISCUSSION

We have designed, expressed, and characterized a variant of AT in which a disulfide bond between the hinge region (E381C) and the adjacent strand on \(\beta\)-sheet A (K222C) effectively prevents the uncoiling of the hinge region upon heparin activation. On a superficial level, the hypothesis that this variant was made to test is simple: that the conformational activation of AT by the pentasaccharide toward inhibition of factors Xa and Xa requires the increased RCL flexibility provided by hinge region expulsion from \(\beta\)-sheet A. However, this study was designed to address the fundamental question in the field: How is AT inhibitory specificity determined? AT is capable of inhibiting most Arg- or Lys-specific proteases and has been shown to be able to inhibit most of the serine proteases involved in blood clotting and fibrinolysis (for a summary see Ref. 35). Because AT circulates at a high concentration in blood, regulation of AT clotting and fibrinolysis (for a summary see Ref. 35). Because AT circulates at a high concentration in blood, regulation of AT

proteases and that the magnitude of pentasaccharide activation reflected how well the RCL of AT corresponded to the substrate consensus sequence (36). Recent studies demonstrating that the activation mechanism is insensitive to the composition of the RCL (15, 37) have led to the current hypothesis that exosite interactions determine AT specificity (12). Thus, the issue of AT inhibitory specificity can be understood in terms of the ability of AT to form a stable Michaelis complex with a protease (Fig. 1), and this in turn depends on the ability to simultaneously form interactions between the active site of the protease and the RCL of AT, and the exosites of AT with the exosites of the protease. This is particularly true in the case of factors IXa and Xa where a several hundred-fold increase in rate of inhibition is stimulated by the binding of AT to the minimal heparin pentasaccharide sequence.

We hypothesized that the AT Michaelis complexes with factors IXa and Xa would be similar to that seen in the crystal structure of HCII with thrombin, and the hypothesis has been explained in detail elsewhere (18). Briefly, it suggests that the exosites required for stabilization of the Michaelis complexes are “pre-formed” on the top of AT, but that they cannot be accessed by the protease while engaging the RCL within its active site, until the hinge region is expelled from \(\beta\)-sheet A. The argument against the “exposure” of new exosites on AT is based on the crystal structures of native and pentasaccharide-bound AT, which show no conformational change in the upper \(\beta\)-barrel domain, where a protease is likely to make contact with AT in a Michaelis complex. The AT-protease contacts in the hypothesized progressive and activated Michaelis complexes would be identical, and the lower progressive rate of inhibition would be due either to the equilibrium position between native and active conformations, or an induced-fit mechanism, whereby initial protease contact with the RCL would help extract the hinge from \(\beta\)-sheet A. Similarly, the Michaels complex between AT and thrombin was hypothesized to be identical in the absence and presence of heparin, however, it would not require or benefit from the expulsion of the hinge region. Rather, the unfolding of the hydrogen-bonded turn in the P region of the RCL would be required to allow thrombin to move away from the top of the body of AT to relieve steric clashes with the bulky insertion loops surrounding the active site cleft. This would explain the insensitivity of the rate of thrombin inhibition to the state of the hinge region, which would not be extended (or necessarily expelled) in the Michaelis complex between AT and thrombin.

The hinge region constrained variant of AT (K222C/E381C) thus allows for the direct testing of our hypothesis. As predicted, we found that thrombin inhibition does not in any way rely on hinge region extension. This is consistent with our recent structure of the AT-thrombin-heparin ternary complex, which showed thrombin situated toward the front of AT and the partial incorporation of the hinge region into \(\beta\)-sheet A (33). The contact interface between AT and thrombin in the structure was extensive and supports the idea that the progressive and heparin-activated Michaelis complexes are identical. Another implication of the structure is that hinge region expulsion is not necessary to achieve high affinity binding of AT to heparin. This was also shown crystallographically in the context of the active/latent dimer, which showed small changes in crystallization conditions can determine whether the pentasaccharide-bound active monomer possesses an expelled or a native-like hinge region conformation (32). We were surprised to find that the hinge region constrained AT variant K222C/E381C bound to the heparin pentasaccharide with the same affinity as the single mutant controls, but this finding is less surprising in context of the structural data. It is even possible...
FIG. 6. Molecular models of the progressive and heparin-activated Michaelis complexes formed by AT. The targets of AT are thrombin (A), factor Xa (B) and factor IXa (C) as defined by rates of inhibition and the ability of heparin to accelerate inhibition. The molecular models are based on the data described here and on two crystal structures: HCII Michaelis complex with thrombin, which requires full hinge region extension, and AT Michaelis complex with thrombin and heparin, which is insensitive to hinge region extension. A, the contacts between AT and thrombin are predicted to be identical for the progressive (left) and activated (right) Michaelis complexes. The activated complex is the actual crystal structure of the ternary AT-thrombin-heparin complex. B, the Michaelis complexes between AT and factor Xa utilize different contacts. The progressive Michaelis complex does not require hinge region expulsion and utilizes contacts similar to those found in the AT-thrombin complex, whereas the activated complex is fully dependent on hinge region extension and resembles the HCII-thrombin complex. C, there is predicted to be a single AT-IXa Michaelis complex, in both the absence and presence of the heparin pentasaccharide, which can only be formed when the hinge region of AT is fully extended. The progressive rate is very low, because the majority of native AT is hinge region-constrained, whereas pentasaccharide activation is due to a shift in the equilibrium to the hinge region-expelled conformation.
that thrombin inhibition by AT derives an advantage through the insertion of the hinge region in the Michaeis complex. The stoichiometry of thrombin inhibition by AT is highly sensitive to conditions, so that at low ionic strength heparin effectively converts AT into a thrombin substrate (38). This demonstrates the similarity in rates of RCL insertion ($k_e$) and deaclylation ($k_d$) illustrated in Fig. 1. Incorporation of the hinge region before acylation may provide AT with the head-start required for RCL insertion to beat out deaclylation, so that under physiological conditions the inhibitory pathway predominates.

The effect of constraining the hinge region of AT on factor Xa inhibition is more complex. If the hypothesis had been correct, the progressive and pentasaccharide-activated rates of factor Xa inhibition would have been close to zero for the hinge region constrained AT variant K222C/E381C. However, the effect of the AT double mutant on the basal rate of factor Xa inhibition was perfectly additive, indicating that the hinge region disulfide bond did not perturb the formation of the progressive Michaelis complex with factor Xa. It is concluded that AT in the progressive Michaelis complex with factor Xa is in a native-like, hinge region-inserted conformation, and that the progressive rate of inhibition is not a measure of the equilibrium position between the native and activated conformation in the absence of heparin. Nor does it reflect an induced fit mechanism. Rather, the data suggest that factor Xa utilizes exosite contacts that are available on native, hinge region-inserted AT. However, the hinge region disulfide bond completely abrogated the pentasaccharide activation of AT toward factor Xa. The absolute nature of this result was particularly satisfying, because it shows that allosteric activation of AT is absolutely dependent on the extension of the hinge region. Furthermore, the predicted exosite on AT can now be reduced to the area accessible to activated wild-type AT, but inaccessible in the context of the K222C/E381C disulfide bond.

Factor IXa inhibition by AT variant K222C/E381C is consistent with the original hypothesis that one set of exosite contacts is shared by both the progressive and activated Michaelis complexes and that formation of the Michaelis complex requires the extension of the hinge region. As would be predicted from the hypothesis, the constraint imposed upon the hinge region by the disulfide bond between K222C and E381C effectively reduces the rate of factor IXa inhibition to the limit of detection, both in the absence and presence of the pentasaccharide. Thus, the low progressive rate of factor IXa inhibition by AT reflects either the equilibrium position between native and activated or the energetic cost of inducing hinge region extension in the absence of the heparin pentasaccharide. If the progressive rate is determined by the equilibrium in the absence of pentasaccharide, the fraction of AT in the activated conformation can be calculated from the rates of inhibition obtained here. If fully activated AT inhibits factor IXa at a rate of $4 \times 10^4$ M$^{-1}$s$^{-1}$, and the native (hinge region constrained) AT inhibits IXa at the maximal estimated rate of $0.5$ M$^{-1}$s$^{-1}$, then the observed progressive rate constant of 96 M$^{-1}$s$^{-1}$ indicates that no more than one AT molecule in 400 (0.24%) is in the activated conformation in the absence of the pentasaccharide (assuming that at saturating pentasaccharide 100% of AT is hinge region-expelled).

In conclusion, we have determined the requirement of hinge region extension in the formation of the Michaelis complexes between AT and its targets, factor IXa, factor Xa, and thrombin. The properties of an AT variant with its hinge region constrained by an engineered disulfide bond reveal three distinct mechanisms for regulating the inhibitory activity of AT. Thrombin is wholly insensitive to the conformation of the hinge region of AT and likely forms a single Michaelis complex in the absence and presence of heparin (Fig. 6A). The exosite contacts between AT and thrombin are equally accessible in the absence and presence of heparin, with bridging heparin chains serving to facilitate the encounter of AT and thrombin and to further stabilize the Michaelis complex. These exosites have been revealed by the recent structure of the AT-thrombin-heparin ternary complex (33) and support the conclusions based on the biochemical properties of the hinge region-constrained K222C/E381C AT variant described here. Factor Xa is predicted to have two different Michaelis complexes in the absence and presence of the pentasaccharide (Fig. 6B). The progressive rate of factor Xa inhibition was not affected by the presence of the disulfide bond, but hinge region extension was absolutely required for pentasaccharide activation. AT was found to be incapable of factor IXa inhibition without the extension of the hinge region, and thus only one AT-factor IXa Michaelis complex can be formed (Fig. 6C). The Michaelis complexes shown in Fig. 6 are based on the structures of the AT-thrombin-heparin ternary complex and the HCII-thrombin Michaelis complex. Where hinge region extension was not involved the AT-thrombin complex structure was used, and where extension was absolutely required the HCII-thrombin complex was used. These models provide a new starting point for mutagenesis strategies aimed at determining the individual interactions that define AT inhibitory specificity.

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