Antithrombin becomes an efficient inhibitor of factor Xa and thrombin by binding a specific pentasaccharide sequence found on a small fraction of the heparan sulfate proteoglycans lining the microvasculature. In the structure of native antithrombin, the reactive center loop is restrained due to the insertion of its hinge region into the main $\beta$-sheet A, whereas in the heparin-activated state the reactive center loop is freed from $\beta$-sheet A. In both structures, hinge region residue Glu-381 makes several stabilizing contacts. To determine the role of these contacts in the allosteric mechanism of antithrombin activation, we replaced Glu-381 with an alanine. This variant is less active toward its target proteases than control antithrombin, due to a perturbation of the equilibrium between the two forms, and to an increase in stoichiometry of inhibition. Pentasaccharide binding affinity is reduced 4-fold due to an increase in the off-rate. These data suggest that the main role of Glu-381 is to stabilize the activated conformation. Stability studies also showed that the E381A variant is resistant to continued insertion of its reactive center loop upon incubation at 50 °C, suggesting new stabilizing interactions in the native structure. To test this hypothesis, and to aid in the interpretation of the kinetic data we solved to 2.6 Å the structure of the variant. We conclude that wild-type Glu-381 interactions stabilize the activated state and decreases the energy barrier to full loop insertion.

The pharmacological basis of heparin anticoagulant therapy is the activation of AT toward the inhibition of factor Xa and thrombin. The mechanism by which AT is activated by heparin is a subject of much interest, both due to its importance as a therapeutic target and because it serves as a model for GAG-binding proteins and for protein allostericity in general (for review see Ref. 3).

AT binds heparin by an induced-fit mechanism (4) in an apparent three step process (Fig. 1a), where the formation of an initial weak complex is followed by the recognition of the correct pentasaccharide sequence that induces a conformational change in the heparin binding region (5). This intermediate is then competent to expel the reactive center loop (RCL) and close the main $\beta$-sheet A into the five-stranded form, which resembles all other known “active” serpin structures. The conformational changes of each step have been identified crystallographically (5–9). The native form of AT is poorly active as an inhibitor due to the partial insertion of the RCL as strand four in $\beta$-sheet A. The constraints on the RCL restrict its flexibility, partially obstruct the crucial P1 Arg, and may cover protease recognition exosites (1). In addition, the native six-stranded form is only capable of weak interaction with the pentasaccharide, and only after expulsion of the RCL is the high-affinity AT-pentasaccharide complex formed. The activated complex has undergone several conformational changes including: the formation of helix P at the N terminus of helix D; the C-terminal elongation of helix D; the rotation and elongation of the N terminus of helix A; the expulsion of the hinge region of the RCL from $\beta$-sheet A; and, the closing of the gap between strands 3 and 5 to form a fully hydrogen bonded five-stranded $\beta$-sheet A. From a recent structure of the heparin-bound intermediate, $I_2$, it appears that helix D elongation serves to lock $\beta$-sheet A in the five-stranded form. One major question concerning the allosteric mechanism of heparin binding by AT is how the conformational change is propagated from the heparin binding site to the hinge region of the RCL to effect its expulsion.

A comparison of the interactions made by the hinge region residues in the active and activated structures of AT (Fig. 1, b and c) suggests that Glu-381, at position P13 (13 residues N-terminal to the reactive center P1 Arg, nomenclature of Schechter and Berger (10)) plays an important role, both in stabilizing the native, partially inserted state, and the activated, five-stranded state (11). Additional support for this hypothesis was provided by the structure of a P14 Cys mutant derivatized by a fluorescein moiety (12). The fluorescein derivative was fully activated in solution, but assumed the native fold in the crystal. The effect of the fluorescein was thus not to exclude the hinge region from inserting into $\beta$-sheet A, but to destabilize the native conformation by breaking the interactions normally made by P13 Glu (Fig. 1d). The presence of the
fluorescein also appeared to perturb the heparin binding region through a change in side chain positions. This lead to the hypothesis of long-range electrostatic communication between the heparin binding region and the hinge region, with P13 Glu serving to sense the electrostatic environment. The recent structure of the heparin-bound intermediate AT conformer (5) support these hypotheses.

TABLE I

| Penta | Factor Xa | Thrombin |
|-------|-----------|----------|
|       | $k_{app}$ | $k_{app}$ | $k_{app}$ |
|       | $\text{s}^{-1}$ | $\text{SI}$ | $\text{SI}$ | $\text{SI}$ |
| S137A | $4.8 \pm 0.1 \times 10^2$ | $0.97 \pm 0.01$ | $4.66 \times 10^4$ | $6.4 \pm 0.6 \times 10^3$ | $1.18 \pm 0.02$ | $7.55 \times 10^2$ |
| E381A | $1.8 \pm 0.1 \times 10^2$ | $1.35 \pm 0.01$ | $2.57 \times 10^4$ | $4.7 \pm 0.1 \times 10^3$ | $2.81 \pm 0.02$ | $6.16 \times 10^3$ |

$^a$ ND, not determined.

$^b$ Stoichiometry of inhibition.

**Fig. 1.** The induced-fit heparin binding mechanism of antithrombin. a. antithrombin (ribbon diagram) interacts with heparin (ball-and-stick) in an apparent three step, four state mechanism. The native state is in the low activity and low affinity conformation with its reactive center loop (yellow and green) partially inserted into the main β-sheet A (red). The specific heparin pentasaccharide interacts with antithrombin primarily via helices A (green) and D (cyan). Although kinetic data fit a model with only one weak-binding intermediate, $I_1$, a recent crystallographic structure suggests that there may be an additional intermediate that has undergone all conformational changes save expulsion of the reactive center loop and helix D elongation, $I_2$. Full activation occurs only after expulsion of the hinge region of the reactive center loop, and the closing of β-sheet A to the five stranded form (Activated). b–e, stereo representations of the main chain and selected side chains of hinge region and surrounding residues reveal important interactions in several crystal forms of antithrombin. b–c correspond to native (α-glycoform, 1e05), pentasaccharide-activated (1e03), S380C-fluorescein-derivatized (1zh), and the heparin-bound intermediate I2 (1nq9), respectively. In native AT (b) the P13 Glu (381) bridges strands 3, 4, and 5A to helix F residues (Arg-197 and Glu-195) through a series of salt-bridges and hydrogen bonds. In the activated conformation (c) strands 3 and 5A have annealed with P13 Glu interacting with strands 2 and 5A and helix F. A fluorescein moiety at the P14 position (d) blocked the native P13 interactions leading to an activated conformation in solution. One of the major conformational responses to heparin binding in the intermediate $I_2$ (e) is the repositioning of Tyr-220 so that it no longer interacts with P13 Glu. Residue numbers are given for some of the residues for clarity.
Antithrombin Allostery and Metastability

4915

The E381A mutant was constructed on the recombinant β-glycoform S137A to reduce glycosylation heterogeneity and facilitate purification though improved heparin affinity (13). S137A (referred to in the text as control) and S137A/E381A (referred to in the text as E381A or P13 Ala variant) variants were made by site-directed mutagenesis of the human antithrombin cDNA in the pMA-Stop plasmid using the Stratagene QuickChange Site-Directed Mutagenesis kit using the appropriate primers for the mutations described. pMA-Stop antithrombin expression plasmids were stably co-transfected into competent baby hamster kidney cells with pRMH140 and pSV2dhfr plasmids using GenePORTER transfection reagent (Gene Therapy Systems, Inc) and selected by resistance to neomycin (Sigma) and methotrexate (Sigma) as previously described (14). Cells were grown to confluence in triple flasks and antithrombin 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 of antithrombin, eluting at around 1.0 and 1.5 NaCl, respectively, as described previously for N135Q antithrombin (15). All experiments were carried out on the high affinity (non-fucosylated) form. Purified material was stored at −80 °C prior to use. Concentration of antithrombin was determined by absorbance at 280 nm and was judged to contain no significant amount of inactive material by its ability to form a stoichiometric covalent complex with thrombin by SDS-PAGE. The concentration of active antithrombin was further verified by stoichiometric titrations of 1 μM antithrombin against increasing known concentrations of physiological pentasaccharide. Active concentrations corresponded to those determined by absorbance at 280 nm for all recombinant and plasma forms studied.

Rates of Inhibition—The rates of inhibition of human factor Xa and human thrombin were determined essentially as previously reported (16), in 20 mM NaPO₄, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000, 0.1% bovine serum albumin, pH 7.4. Briefly, 10 μM each of antithrombin and protease were incubated together at final concentrations of 1 μM and 50 nM, respectively, for between 1 and 10 min at room temperature in the wells of a microtitre plate. At the end of the incubation period, reactions were quenched and residual protease activity was determined by the addition of 200 μL of 300 μM chromogenic substrate (S2222 for factor Xa and S2238 for thrombin) (Chromogenix) and color generation measured at 405 nm on a Thermomax plate reader (Molecular Devices). Observed pseudo first-order rate constants (k(obs)) were taken as the slope of the linear plot of natural log of residual protease activity versus time. The second-order rate constants (k(cat)) were taken as k(cat)/Kd, divided by the total AT concentration. Second order rate constants for the pentasaccharide catalyzed inhibition of factor Xa were derived by determining k(cat)/Kd at a range of pentasaccharide concentrations from 5 to 40 nM. The slope of the linear plot of k(cat)/Kd versus pentasaccharide concentration gave k(cat)/Kd (because Kd was much lower than the total AT concentration) and the y intercept divided by the total AT concentration was taken as k(cat)/Kd.

**TABLE II**

| Equilibrium binding affinity for the pentasaccharide |  |  |  |  |  |
|---|---|---|---|---|
| | I = 0.15 | I = 0.2 | I = 0.3 | Z | K(Ka) | ΔF<sub>max</sub> <sup>a</sup> |
| **K<sub>d</sub>** |  |  |  |  |  |
| S137A | 3.3 ± 1.8 × 10⁻⁶ | 1.1 ± 0.2 × 10⁻⁶ | 5 ± 1 × 10⁻⁹ | 4.89 | 5.6 | 0.40 |
| S137A/E381A | 1.4 ± 0.3 × 10⁻⁶ | 4.2 ± 0.1 × 10⁻⁶ | 2.20 ± 0.07 × 10⁻⁷ | 4.95 | 25.7 | 0.30 |

<sup>a</sup> Derived from double log plot of K<sub>d</sub> versus I, where Z is the number of ionic interactions and K<sub>n,i</sub> is the dissociation constant at 1 M NaCl and provides a measure of the non-ionic contribution to binding.

<sup>b</sup> From emission spectra.
Stoichiometries of Inhibition.—The stoichiometries of antithrombin inhibition of factor Xa and thrombin were determined in the presence and absence of physiological pentasaccharide. Protease (100 nM) was incubated for at least 3 h at room temperature with increasing concentrations of antithrombin from 5 to 160 nM (with 100 nM physiological pentasaccharide when present). Residual protease activity was determined by the addition of 200 nM of 300 nM chromogenic substrate, as above. The residual protease concentration was plotted against the antithrombin/protease ratio, and the stoichiometry of inhibition was taken as the x intercept.

Steady-State Pentasaccharide Binding—Equilibrium dissociation constants ($K_d$) were determined essentially as described previously (17). Briefly, the intrinsic fluorescence of antithrombin (0.025–0.1 μM) interacting with increasing concentrations of pentasaccharide was measured at 340 nm on a PerkinElmer Life Sciences 50B spectrofluorimeter, exciting at 280 nm and using bandwidths of 2.5 nm for both excitation and emission. All solutions were made up in 50 mM Tris, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4 (I = 0.15), and titrations were carried out at room temperature. Fluorescence was recorded by averaging 100 measurements taken during 1-s intervals for each addition of pentasaccharide. Data were fitted as before. Dissociation constants were also determined at ionic strengths of $I = 0.2$ and $I = 0.3$ by adjusting the NaCl concentration in the same buffer. Fluorescence emission spectra were collected on a 1 μM AT samples both before and after addition of saturating concentrations of pentasaccharide (2.5 μM), exciting at 280 nm. Reported errors are standard deviation from at least two determinations.

Rapid Binding Kinetics—Stopped-flow experiments were conducted to determine the rates of pentasaccharide binding, essentially as reported previously (4). All reactions were carried out in $I = 0.15$ buffer on a BioLogic MOS-450 stopped-flow fluorimeter. Rapid injections of antithrombin and pentasaccharide at a range of concentrations from 4 nM to 30 μM were made (always in at least 5-fold molar excess of pentasaccharide to ensure pseudo first-order conditions), and intrinsic fluorescence above 320 nm was detected, exciting at 280 nm. The lower concentration limit of 0.8 nM antithrombin and 4 nM pentasaccharide was determined for the E381A variant, because due to its lower maximal signal and increased $K_d$, the signal was smaller than that observed for the control. The inset of Fig. 3b illustrates the quality of the observed trace and fit for the E381A variant, under the lowest concentration conditions. The rate determined for each condition was the mean of at least 6 (and up to 30) repeat shots. The linear plot of rate versus pentasaccharide concentration at the low range of pentasaccharide concentrations (4 nM-150 nM) provided the on-rate ($k_{on}$) and the apparent off-rate ($k_{off}$). Values for $k_{on}$ and $K_d$ were determined from the non-linear fit of the plot of rate versus pentasaccharide concentration for the full range of pentasaccharide concentrations, as before. $K_d$ is the initial rapid dissociation constant preceding the conformational change step defined by $k_{off}$ (equation in inset of Fig. 3a). Off-rates were also measured directly by rapid dilution experiments, diluting 33 or 120 nM of complex, for control and variant, respectively, between 5- and 10-fold. Under these conditions (initial complex concentration >10-fold higher than the $K_d$) the observed rate was insensitive to initial or final complex concentrations. The value taken for $k_{off}$ was derived from the average of 20 runs.

Rate of Latency Transition—Antithrombin was incubated under conditions which limit polymer formation as before (18), namely 100 μg/ml at 50 °C in 50 mM NaPO4, 40% glycerol, pH 6.0. The antithrombin was pre-treated with neuraminidase to remove sialic acid from the carbohydrate, as before (5). At indicated times samples were removed, snap frozen, and stored at −80 °C prior to assay for thrombin inhibitory activity and on native PAGE.

Structure Determination—Mutant antithrombin was crystallized as a dimer consisting of S137A/E381A antithrombin in its native conformation and plasma-derived antithrombin in its latent conformation. Plasma derived an antithrombin was purified as described previously.

![Figure 3](http://www.jbc.org)

**TABLE III**

|            | $K_d$ | $b_2$ | $h_4$ | $k_{on}$ | $h_{off}$ | $K_{eq}$ |
|------------|-------|-------|-------|----------|-----------|---------|
|            | μM    | s⁻¹   | μM.s⁻¹ | s⁻¹      | s⁻¹       | M       |
| S137A      | 4.9 ± 0.8 | 374 ± 5.4 | 82 ± 4 | 0.58 ± 0.23 | 0.49 ± 0.04 | 6.00 × 10⁻⁹ |
| S137A/E381A | 3.4 ± 0.5 | 378 ± 3.8 | 81 ± 10 | 2.21 ± 0.59 | 1.50 ± 0.03 | 1.85 × 10⁻⁸ |

$^a$ Taken from the y intercept of the linear plot of $k_{obs}$ versus low pentasaccharide concentrations.

$^b$ Derived directly from rapid dilution experiments.

$^c$ Calculated from $k_{off}$ and $h_{off}$. 

Downloaded from http://www.jbc.org by guest on July 25, 2018
Antithrombin Allostery and Metastability

RESULTS

Kinetics of Protease Inhibition—The hypothesis that P13 Glu interactions affect the equilibrium between the native and activated conformations is readily testable by determining the rates of factor Xa inhibition. Unlike thrombin, whose inhibition by AT is activated only 2-fold in the presence of the pentasaccharide, factor Xa inhibition is exquisitely sensitive to pentasaccharide binding, and results in an approximate 100-fold increase in rate of inhibition by AT (23, 24). Rates and stoichiometries of human factor Xa and thrombin inhibition are given in Table IV. At first glance the E381A/S137A mutant (also referred to as E381A and P13 Ala variant) inhibits thrombin more slowly than does the control S137A; however, when the stoichiometries of inhibition are taken into account, there is no difference in rate of thrombin inhibition. The stoichiometry of inhibition for the control was indistinguishable from one as expected, but for the P13 Ala variant it was slightly elevated.

As we had already verified that all of the mutant was active by SDS-PAGE and stoichiometric titrations with known concentrations of pentasaccharide, the results indicated a slower RCL incorporation after initial cleavage by thrombin. In the presence of the pentasaccharide, however, the stoichiometry returned to the control value.

The rates of factor Xa inhibition were determined in the presence of the pentasaccharide. Thus, breaking the native P13 Glu interactions affects the equilibrium between the native and activated conformations is readily testable by determining the rates of factor Xa inhibition. Unlike thrombin, whose inhibition by AT is activated only 2-fold in the presence of the pentasaccharide, factor Xa inhibition is exquisitely sensitive to pentasaccharide binding, and results in an approximate 100-fold increase in rate of inhibition by AT (23, 24). Rates and stoichiometries of human factor Xa and thrombin inhibition are given in Table IV. At first glance the E381A/S137A mutant (also referred to as E381A and P13 Ala variant) inhibits thrombin more slowly than does the control S137A; however, when the stoichiometries of inhibition are taken into account, there is no difference in rate of thrombin inhibition. The stoichiometry of inhibition for the control was indistinguishable from one as expected, but for the P13 Ala variant it was slightly elevated.

As we had already verified that all of the mutant was active by SDS-PAGE and stoichiometric titrations with known concentrations of pentasaccharide, the results indicated a slower RCL incorporation after initial cleavage by thrombin. In the presence of the pentasaccharide, however, the stoichiometry returned to the control value.

The rates of factor Xa inhibition were determined in the presence of the pentasaccharide. Thus, breaking the native P13 Glu interactions affects the equilibrium between the native and activated conformations is readily testable by determining the rates of factor Xa inhibition. Unlike thrombin, whose inhibition by AT is activated only 2-fold in the presence of the pentasaccharide, factor Xa inhibition is exquisitely sensitive to pentasaccharide binding, and results in an approximate 100-fold increase in rate of inhibition by AT (23, 24). Rates and stoichiometries of human factor Xa and thrombin inhibition are given in Table IV. At first glance the E381A/S137A mutant (also referred to as E381A and P13 Ala variant) inhibits thrombin more slowly than does the control S137A; however, when the stoichiometries of inhibition are taken into account, there is no difference in rate of thrombin inhibition. The stoichiometry of inhibition for the control was indistinguishable from one as expected, but for the P13 Ala variant it was slightly elevated.

As we had already verified that all of the mutant was active by SDS-PAGE and stoichiometric titrations with known concentrations of pentasaccharide, the results indicated a slower RCL incorporation after initial cleavage by thrombin. In the presence of the pentasaccharide, however, the stoichiometry returned to the control value.
side chain interaction in the native and heparin-bound states only marginally affects the rate of factor Xa inhibition. The effect is real, however, suggesting that in both the absence and presence of the pentasaccharide the equilibrium between the native and activated conformations has been perturbed toward the left.

Steady-State Pentasaccharide Binding Studies—Consistent with the conclusions drawn from the protease inhibition data, the intrinsic fluorescence spectra of the P13 Ala variant indicate a higher population of native-like intermediate conformer (I_2 in Fig. 1) at saturation concentrations of the pentasaccharide, relative to the control. Antithrombin normally undergoes a 40% enhancement of intrinsic fluorescence when bound to the pentasaccharide (17). The fluorescence spectra of the control S137A AT showed the 40% enhancement, but the P13 Ala variant consistently gave a lower fluorescence enhancement of 30%. Normalized spectra (Fig. 2a) indicate that the lower fluorescence enhancement for the P13 Ala variant is not due to a change in initial fluorescence, but a reduction in the quantum yield of the pentasaccharide-bound state.

Intrinsic fluorescence change upon titration of the pentasaccharide was followed to determine steady-state dissociation constants at physiological ionic strength (Fig. 2b). The low protein concentrations required for the determination of the K_d results in variable maximal fluorescence maxima, but the fluorescence maximum for the P13 Ala variant was always significantly lower than that of the control. At physiological ionic strength the P13 variant bound the pentasaccharide ~4-times more weakly than the control (Table II). To determine if the cause of the reduced heparin affinity was a weakening of ionic or non-ionic interactions, K_d at two elevated ionic strengths were also determined and plotted versus ionic strength on a double log scale (25, 26) (Fig. 2c). The slopes were identical, indicating that the number of ionic interactions between AT and the pentasaccharide is conserved, but the y intercepts were significantly different for the P13 variant and the control (Table II). The y intercept is related to the dissociation constant at 1 M NaCl, and is referred to in the table as K_{NI}, or the non-ionic contribution to the dissociation constant. Thus the substitution of an alanine for glutamate at P13 in AT results in a loss of affinity for heparin due to a reduced non-ionic contribution, and an equilibrium position at saturation resulting in a smaller fraction of AT in the fully activated, five-stranded form. Together these data suggest that the interactions of P13 Glu are less important for the stabilization of the native structure than the activated structure.

Pentasaccharide Binding Rates—To determine which step of the pentasaccharide binding mechanism is perturbed by the P13 Ala mutation, rapid kinetic studies were undertaken. The kinetic model assumes a two step binding mechanism where the first is rapid, fluorescently silent and of weak affinity, and the second involves a conformational change in AT leading to the fluorescence signal (4). (Fig. 1a, where I_1 and I_2 cannot be distinguished). Conditions are maintained pseudo first-order in

![Stereo representations of Cα traces of AT colored according to root mean squared deviation from native αAT.](https://example.com/fig5.png)

- **a.** The Cα trace of the P13 Ala (ball) variant reveals surprisingly profound main chain differences when compared with the α-glycoform of AT. Although perturbation of the hinge region might be expected, the effect seems to have propagated to the heparin binding region. To determine which conformational changes are significant, and which reflect the flexible nature of the molecule, the structure of the β-glycoform compared with the α-form is shown in b. The color scheme reflects the RMSD from light gray to red for 0–3 Å. The distance between Lys-220 and -139 Cα is shown as a dashed green line, and it increases from 5.1 to 7.8 Å in response to the E381A mutation.
the pentasaccharide, and the hyperbolic plot of the observed rate constant versus pentasaccharide concentration (Fig. 3a) provides values for the initial dissociation constant, $K_d$, and the rate constant for the forward conformational change, $k_+$ (equation inset of Fig. 3a). At low pentasaccharide concentration the plot is linear, with the slope equal to the on-rate, $k_{on}$, and the y intercept equal to $k_{\text{off}}$, and is indistinguishable from the off-rate (Table III). In most of the reported cases where an AT mutation has affected the affinity for heparin the off-rate accounts for the majority of the observed effect (27–32). Obtaining accurate off-rates is difficult because it is necessary to extrapolate to the y intercept corresponding to 0 M pentasaccharide from 1 M pentasaccharide, due to the sensitivity of the instrument and the need to maintain pseudo first-order conditions in pentasaccharide. In addition, the extrapolated value is very close to zero, so negative values, with errors in excess of the values, are often obtained. We have improved the accuracy of the values obtained from the extrapolation by using pentasaccharide concentrations to 4 mM (Fig. 3b and inset). We have also determined off-rates from rapid dilution experiments (Fig. 3c), which agreed with those obtained from the linear extrapolation (Table III). The increase in $K_d$ for the E381A variant is thus due solely to an increase in rate of the reverse conformational change, indicating that P13 Glu interactions in native AT do not contribute significantly to the energy barrier to loop expulsion, and that the role of Glu-381 in the heparin binding mechanism is to help lock AT in the activated conformation.

Stability Studies—Because serpins are metastable in their native state it is important to assess the effect of a mutation on both the thermodynamic and the kinetic stability. Melting studies monitoring the CD signal at 222 nm showed no difference in $T_m$ for the control and the P13 Ala mutant (data not shown). This, coupled with the identical initial fluorescence spectra and only marginally altered rates of factor Xa inhibition, suggests that the native conformation of E381A AT is nearly identical to that of the wild-type, and that the mutation has no significant effect on the thermodynamic stability of the native state. Normally there is a correlation between mutations which destabilize the native state of AT and the propensity of AT to convert to the hyperstable latent form (27, 33) (Fig. 4a), or to polymerize. We determined the effect of the P13 Ala substitution on the kinetic stability of AT by incubating at 50 °C under conditions where polymerization is inhibited. The conversion to the inactive hyperstable latent conformer was followed by loss of inhibitory activity and by increased electrophoretic mobility on native PAGE (Fig. 4, b and c). We found that the P13 Ala variant was resistant to conversion to the latent form. After 3 days at 50 °C no activity remained for the control and 82% activity remained for the variant (Fig. 4b). The loss of control activity was confirmed to be due to full conversion to the latent conformation by native PAGE (Fig. 4c). Although the actual magnitude of the increase in activation energy on the reaction pathway from native to latent cannot be estimated from these data, it is clear the substitution of Ala for Glu at P13 has a dramatic effect on the kinetic stability of AT.

Structural Studies—To determine the structural bases behind the effect of the P13 E→A mutation on the heparin binding mechanism and metastability of AT, we crystallized the variant and solved its structure by x-ray crystallography (Table IV). The overall fold is similar to native antithrombin (α-glycoform, 1e05) with a Ca root mean squared deviation (RMSD) of 1.06 Å and a maximal deviation of 6.40 Å. This compared with the Ca RMSD for the α and β glycoforms of 0.83 Å with a maximal deviation of 7.45 Å, indicates a slightly greater overall deviation and that some inherently flexible regions exist. The Ca trace of the structure of the E381A variant colored according to RMSD with αAT is shown in Fig. 5a, and that of βAT colored according to RMSD with αAT is shown in Fig. 5b. The most flexible regions within the two structures correspond to the N-terminal loop from residue 5 to 25 and the N-terminal loop of helix A starting at residue 40. The rest of βAT is nearly indistinguishable from αAT, with the exception of a small movement in the flexible loop C-terminal to helix D where the glycosylation site absent in βAT is found (residue 135). In this context, the movement of Ca atoms in response to the substitution at P13 is dramatic. Predictably, the hinge region moves in response to the breaking of the contacts normally formed by Glu at P13, but in addition, the entire heparin binding site has been significantly rearranged in response to the mutation. The back-bone structural changes obviously originate at the hinge region, and then are apparently propagated to the heparin binding region through the tops of strands 3 and 2A. Indeed, one of the most unusual structural features is the parting of the strands between residues Lys-139 and Lys-222, resulting in the loss of two β-sheet hydrogen bonds (Figs. 5a and 6a). The electron density of the gap between strands 2 and 3A and the hinge region (Fig. 6, a and b) demonstrates the unequivocal nature of these structural features.

**DISCUSSION**

As for the other serpins, AT depends on its metastability to inhibit serine proteases. The serpin mechanism requires a finely tuned balance to allow folding into the five-stranded active conformation while also permitting the rapid, thermodynamically driven loop insertion required to crush and inactivate the protease (34). If the energy barrier to full loop insertion is too high, loop insertion will not occur at a rate sufficient for protease inhibition, and if the energy barrier is too low, then the serpin will be inactivated through conversion to the latent conformer. In this context, AT is even more precariously balanced as in its native conformation the RCL is partially pre-inserted into β-sheet A. The required loosening up of the top of β-sheet A helps to reduce the energy barrier to the conversion to the hyperstable latent form, and consequently AT converts spontaneously to the latent form at ambient temperatures. Heparin activation results in the reversal of the pre-insertion and a conversion to the normal native serpin fold. At the center of this mechanism is a glutamate in the hinge region of the RCL at position P13. As Fig. 1b illustrates, in native AT P13 Glu is centrally involved in a hydrogen-bonding network which binds together strands 3 and 5A, the overlying helix F and the hinge region of the RCL. In the active conformation, P13 Glu binds strand 2 and 3A together with helix F, thus predictably stabilizing the activated, five-stranded form. It is thus easy to hypothesize that P13 may play a role in the heparin activation mechanism, but harder to predict what effect its substitution may have. This study clearly indicates that the P13 Glu interactions in the activated state are crucial to achieve full activation in the presence of saturating pentasaccharide, but are perhaps less critical for stabilizing the native conformation.

The conclusion drawn from the biochemical data is that the E381A mutation perturbs the equilibrium between the native and activated conformations in both the presence and absence of pentasaccharide. The 2-fold lower rate of factor Xa inhibition for uncatalyzed and pentasaccharide catalyzed reactions indicates that the activated state is less populated. This can be explained by the heparin binding data, which show that the activated conformation is less stable due to an increase in $k_{\text{off}}$. Why is the activated, five-stranded conformation destabilized for the E381A variant? The obvious reason is the loss of stabilizing salt-bridge between P13 Glu and Lys-139 on strand 2A. The structure of the variant, however, would indicate that in
addition to creating stabilizing interactions, P13 Glu interposes between basic groups to prevent destabilizing contacts. It was surprising to find the position of the main chain of Lys-139 perturbed to such a degree as to break two /H9252 -sheet hydrogen bonds. The driving force for this may be the apposition of lysines 222 and 139 in the correctly hydrogen-bonded sheet A. Although Lys-222 does not directly interact with Glu-381 in the native structure, they are around 6.5 Å apart with Glu-381 interposing between Arg-197 and Lys-222. The electrostatic potential of the region will be significantly different without the neutralizing effect of Glu-381 in the native structure, but in the activated structure Glu-381 makes direct contact with Lys-139 and interposes directly between Lys-222, Arg-197, and Lys-139.

Although Glu-381 makes favorable contacts in the native state, the effect of the Glu→Ala mutation is an increase in the kinetic stability of native AT. This is manifested as a resistance toward full loop insertion. In the absence of protease it converts to latent at a very slow rate relative to control, but in the presence of protease there is also evidence that loop insertion is slowed. The stoichiometries of inhibition of factor Xa and thrombin are elevated relative to the control in the absence, but not the presence of the pentasaccharide. Thus, if the loop is expelled before interaction with protease, insertion of the reactive center loop is faster than deacylation, but if the hinge region is pre-inserted then contacts are formed which significantly slow full loop insertion after cleavage of the reactive center bond. The structure of the E381A variant suggests what the compensatory interactions of the P13 Ala are. Fig. 6, b and c, show the hinge region of E381A in the native state. The P13

Fig. 6. Stereo representations of regions significantly changed due to the E381A mutation. a, electron density (contoured at 1σ) of the top of strands 2 and 3A reveal a loss of two anti-parallel /H9252 -sheet hydrogen bonds between Lys-139 and -222. b, electron density of the hinge region (P18 to P12) demonstrates the certainty in the position of the main chain oxygen of P13 Ala, as well as the new hydrogen bonds responsible for the resistance of E381A to the latency transition. c, a comparison of hinge region interactions for aAT (magenta with black dashed lines for hydrogen bonds) and the E381A variant (yellow with green dashed lines for hydrogen bonds) summarizes the effect of the E381A mutation. Although the position of the glutamate side chain in the breach between strands for wild-type AT forms stabilizing interactions, it also prevents the formation of a main chain hydrogen bond seen for the variant. It is anticipated that the main chain hydrogen bond slows significantly further loop insertion, and that the purpose of positioning the glutamate side chain in the breach is to prevent P13 main chain hydrogen bond formation. The only other structure of a serpin with this conformation, heparin cofactor II, also positions its P13 side chain into the breach with a similar resulting main chain conformation.
main chain oxygen is flipped relative to the position it adopts in aAT, allowing it to make a strong hydrogen bond to the side chain of Asn-376 (Fig. 6c). The side chain of Asn-376 is flipped in the E381A structure and the hydrogen bond with the P13 carbonyl oxygen is strengthened by interactions between the side chain oxygen of Asn-376 and the main chain amides of residues 378 and 379. This hydrogen bond network may account for the kinetic stability of the E381A variant because loop insertion cannot proceed further without breaking these interactions. Thus the wild-type interactions may serve only to stabilize the interactions with Asn-376 seen in the E381A structure and the hydrogen bond with the P13 chain of Asn-376 (Fig. 6).

Evidence for this conclusion is provided by the only other insertions in the E381A structure and the hydrogen bond with the P13 Gln side chain oxygen of Asn-376 and the main chain amides of carbonyl oxygen is strengthened by interactions between the side chain of Asn-376 and the hydrogen bond with the P13.

Inserted cannot proceed further without breaking these interactions. Thus the wild-type interactions may serve only to stabilize the interactions with Asn-376 seen in the E381A structure and the hydrogen bond with the P13.

Acknowledgments—We thank thank Maurice Petitou for the gift of the pentasaccharide.

REFERENCES
1. Olson, S. T., and Chuang, Y. J. (2002) Trends Cardiovasc. Med. 12, 331–338
2. Thunberg, L., Backstrom, G., Wasteson, A., Robinson, H. C., Ogren, S., and Lindahl, U. (1982) J. Biol. Chem. 257, 15073–15079
3. Huntington, J. A. (2003) J. Biol. Chem. 278, 16215–16220
4. Olson, S. T., Bjork, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1981) J. Mol. Biol. 153, 587–606
5. Johnson, D. J., and Huntington, J. A. (2003) Biochemistry 42, 8712–8719
6. Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J., Groenendaal, P. D., and Hol, W. G. (1994) Nat. Struct. Biol. 1, 48–54
7. McCoy, A. J., Pei, X. Y., Skinner, R., Abrahams, J. P., and Carrell, R. W. (2003) J. Mol. Biol. 326, 825–833
8. Carrell, R. W., Stein, P. E., Fermi, G., and Wardell, M. R. (1994) Structure. 2, 257–270
9. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14683–14688
10. Scheckter, L., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162
11. Whistock, J. C., Pike, R. N., Jin, L., Skinner, R., Pei, X. Y., Carrell, R. W., and Leck, A. M. (2000) J. Mol. Biol. 301, 1287–1305
12. Huntington, J. A., McCoy, A., Belzar, K. J., Pei, X. Y., Gettins, P. G., and Carrell, R. W. (2000) J. Biol. Chem. 275, 15377–15383
13. Mushanu, A., Zhou, A., Carrell, R. W., and Huntington, J. A. (2003) Blood 102, 4028–4034
14. Zettlemoe, G., Wirth, M., Hauser, H., and Kupper, H. A. (1988) Behring Inst. Mitt. 26–34
15. Garone, L., Edmunds, T., Hanson, E., Bernasconi, R., Huntington, J. A., Meagher, J. L., Fan, B., and Gettins, P. G. (1996) Biochemistry 35, 8881–8889
16. Olson, S. T., Bjork, I., and Shore, J. D. (1993) Methods Enzymol. 222, 525–559
17. Olson, S. T., and Shore, J. D. (1981) J. Biol. Chem. 256, 11065–11072
18. Zhou, A., Stein, P. E., Huntington, J. A., and Carrell, R. W. (2003) J. Biol. Chem. 278, 15116–15122
19. Zhou, A., Huntington, J. A., and Carrell, R. W. (1999) Blood 94, 3388–3396
20. Krapf, P. J. (1991) J. Appl. Crystalogr. 24, 946–950
21. Eason, R. M. (1997) J. Mol. Graph. Model. 15, 122
22. Merritt, E. A., and Murphy, M. E. P. (1994) Acta Crystallographica D50, 869–873
23. Olson, S. T., Bjork, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) J. Biol. Chem. 267, 12528–12538
24. Turk, B., Briedtis, I., Bock, S. C., Olson, S. T., and Bjork, I. (1997) Biochemistry 36, 6602–6609
25. Record, M. T. J., Lohman, M. L., and De Haseth, P. (1976) J. Biol. Chem. 251, 145–158
26. Belzar, K. J., Dafforn, T. R., Petitou, M., Carrell, R. W., and Huntington, J. A. (2000) J. Biol. Chem. 275, 8733–8741
27. Beauxchamp, N. J., Pike, R. N., Daly, M., Butler, L., Makris, M., Dafforn, T. R., Zhou, A., Fitton, H. L., Preston, F. E., Peake, I. R., and Carrell, R. W. (1998) Blood 92, 2709–2716
28. Desai, U., Swanson, R., Bock, S. C., Bjork, I., and Olson, S. T. (2000) J. Biol. Chem. 275, 18976–18984
29. Arocas, V., Bock, S. C., Raja, S., Olson, S. T., and Bjork, I. (2001) J. Biol. Chem. 276, 43809–43817
30. Pike, R. N., Potempa, J., Skinner, R., Fitton, H. L., McGraw, W. T., Travis, J., Owen, M., Jin, L., and Carrell, R. W. (1997) J. Biol. Chem. 272, 18652–18658
31. Arocas, V., Bock, S. C., Olson, S. T., and Bjork, I. (1999) Biochemistry 38, 10196–10204
32. Belzar, K. J., Zitt, A., Carrell, R. W., Gettins, P. G., and Huntington, J. A. (2002) J. Biol. Chem. 277, 8551–8558
33. Quinsey, N. S., Fitton, H. L., Coughlin, P., Whistock, J. C., Dafforn, T. R., Carrell, R. W., Bottomley, S. P., and Pike, R. N. (2000) Biochemistry 42, 10169–10173
34. Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) Nature 407, 923–926
The Influence of Hinge Region Residue Glu-381 on Antithrombin Allostery and Metastability
Daniel J. D. Johnson and James A. Huntington

J. Biol. Chem. 2004, 279:4913-4921.
doi: 10.1074/jbc.M311644200 originally published online November 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M311644200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 33 references, 15 of which can be accessed free at http://www.jbc.org/content/279/6/4913.full.html#ref-list-1