Antithrombin is unique among the serpins in that it circulates in a native conformation that is kinetically inactive toward its target proteinase, factor Xa. Activation occurs upon binding of a specific pentasaccharide sequence found in heparin that results in a rearrangement of the reactive center loop and activation. The crystal structure of an activated antithrombin variant, N135Q S380C-fluorescein (P14-fluorescein), in order to see how full activation is achieved in the absence of heparin and how the structural effects of the substituents in the hinge region are translated to the heparin binding region. The crystal structure resembles native antithrombin except in the hinge and heparin binding regions. The absence of global conformational change allows for identification of specific interactions centered on Glu881 (P13), that are responsible for maintenance of the solution equilibrium between the native and activated forms and establishes the existence of an electrostatic link between the hinge region and the heparin binding region. A revised model for the mechanism of the allosteric activation of antithrombin is proposed.

Antithrombin is unique as a proteinase inhibitor because it requires allosteric activation. Its cofactor, heparin, binds antithrombin via a specific pentasaccharide domain that accounts for its anticoagulant activity. Antithrombin plays a critical role in the prevention of thrombosis by inhibiting the final two steps in the coagulation cascade, factor Va and thrombin. The tight regulation of antithrombin involves the following: 1) maintenance of an inactive, native pool in plasma at 2.3 μM; 2) localization to the vascular endothelium through binding to glycosaminoglycans; 3) conformational activation; 4) ability to undergo a massive conformational rearrangement upon reaction with proteinase; and 5) release from glycosaminoglycan upon formation of complex with proteinase. The complexity of the role of antithrombin in hemostasis is illustrated by the wide range of antithrombin mutations that lead to thromboembolic disorders (2).

Antithrombin is a member of the serpin superfamily of serine proteinase inhibitors and maintains the overall serpin fold (3, 4). It is thus capable of undergoing a dramatic conformational rearrangement upon attack by a cognate proteinase at position P1–P1′ (nomenclature of Schechter and Berger (1)) where the surface-exposed reactive center loop is incorporated as strand four in the central β-sheet A (5). The loop-inserted form is significantly more stable than the native form of serpins (6, 7), and it is believed that the energy required to stabilize the inhibitory complex with a proteinase is supplied from loop insertion (8). All of the known structures of native serpins maintain a flexible, exposed reactive center loop containing a proteinase-accessible P1 side chain, with the exception of antithrombin (3, 4, 9–14). The reactive center loop of native antithrombin is constrained by the insertion of hinge region residues P15 and P14 as s4A, resulting in interactions between the P1 Arg side chain and the body of the serpin (Fig. 1).

The conformation of the reactive center loop of native antithrombin is the structural basis of its marginal rate of factor Xa inhibition. The structure of pentasaccharide-bound antithrombin has recently been solved by x-ray crystallography (Fig. 1) and reveals expulsion of the hinge region and secondary structural changes in the heparin binding region consisting of helix A and D elongation and helix P formation (15). Biochemical studies have indicated that activation is ultimately the result of the reorientation of the cognate P1 Arg from interacting with the main body of the protein to pointing out toward a potential attacking proteinase (16). The reorientation of the P1 side chain is not seen in the pentasaccharide-bound structure, presumably due to contacts between the reactive center loop of the active monomer and its latent counterpart.

Activation can also be effected by the forced expulsion of the hinge region through substitution of the P14 Ser with bulky or charged residues. This has been shown using a P14 Ser → Trp, which gave a 3-fold increase in rate of reaction with factor Xa (17), and for a P14 Ser → Cys variant derivatized with fluorescein that was fully activated toward factor Xa (460-fold over P14-Cys control) (18). Such variants achieve activation through a destabilization of the native state resulting in a shifting of the equilibrium position between the active and native states (Fig. 1). In contrast, the pentasaccharide alters the equilibrium position by stabilizing the activated state. This is borne out by thermal denaturation studies that show a 10 °C increase in stability for antithrombin bound to the physiological pentasaccharide, a 20 °C increase in stability for antithrombin bound to a synthetic pentasaccharide with a 100-fold higher affinity for antithrombin, and an 8 °C decrease in stability for the pentasaccharide. Antithrombin is unique among the serpins in that it circulates in a native conformation that is kinetically inactive toward its target proteinase, factor Xa. Activation occurs upon binding of a specific pentasaccharide sequence found in heparin that results in a rearrangement of the reactive center loop and activation. The crystal structure of an activated antithrombin variant, N135Q S380C-fluorescein (P14-fluorescein), in order to see how full activation is achieved in the absence of heparin and how the structural effects of the substituents in the hinge region are translated to the heparin binding region. The crystal structure resembles native antithrombin except in the hinge and heparin binding regions. The absence of global conformational change allows for identification of specific interactions centered on Glu881 (P13), that are responsible for maintenance of the solution equilibrium between the native and activated forms and establishes the existence of an electrostatic link between the hinge region and the heparin binding region. A revised model for the mechanism of the allosteric activation of antithrombin is proposed.

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FIG. 1. Ribbon diagrams of native and pentasaccharide-bound antithrombins. The central β-sheet A is in red; the reactive center loop is in yellow; helix D is in cyan; helix A is in green; and the P1 arginine in red space filling. The secondary structural changes in the pentasaccharide bound structure are in yellow, and the pentasaccharide is in a ball-and-stick representation. Glycosaminoglycan binding occurs via the carbohydrate bound structure are in yellow in the absence of glycosaminoglycan is reversed, with k2, and the reverse rate, k−2, is independent. Activation ultimately depends on release of constraints on the P1 Arg loop resulted from a mechanical force effected on strand 2A by the C-terminal elongation of helix D and that expulsion of the reactive center terminus of helix D and that expulsion of the reactive center loop resulted from a mechanical force effected on strand 2A by the C-terminal elongation of helix D. The structure of pentasaccharide-bound antithrombin revealed both helix D elongation and loop expulsion but not, as proposed, through direct interaction between the elongated portion of helix D and the pentasaccharide. This leaves the question of how pentasaccharide binding causes expulsion of the hinge region from β-sheet A. We hypothesize that the D-helix elongation is a consequence of the electrostatic effect of pentasaccharide binding and that expulsion is not effected by a mechanical force but rather a perturbation of surface electrostatics that help determine the equilibrium position between the native and activated states.

The structures of native and pentasaccharide-bound antithrombins, as well as the biochemical studies on P14 variants, provide convincing evidence that activation requires the expulsion of the hinge region. A major question remains concerning the mechanism of allosteric activation of antithrombin. How do the local structural changes caused by pentasaccharide binding result in the expulsion of the hinge region from β-sheet A? It was hypothesized by van Boeckel and colleagues (20), based on the structure of native antithrombin and computer simulations, that binding of the pentasaccharide occurred at the C terminus of helix D and that expulsion of the reactive center loop resulted from a mechanical force effected on strand 2A by the C-terminal elongation of helix D. The structure of pentasaccharide-bound antithrombin revealed both helix D elongation and loop expulsion but not, as proposed, through direct interaction between the elongated portion of helix D and the pentasaccharide. This leaves the question of how pentasaccharide binding causes expulsion of the hinge region from β-sheet A. We hypothesize that the D-helix elongation is a consequence of the electrostatic effect of pentasaccharide binding and that expulsion is not effected by a mechanical force but rather a perturbation of surface electrostatics that help determine the equilibrium position between the native and activated states. This hypothesis is tested here by determining the structure of an antithrombin variant, N135Q, S380C, that is fully activated in solution in the absence of pentasaccharide due to the presence of a bulky fluorescein moiety bound at the P14 position (18). We specifically address the questions of how the solution equilibrium between the native and activated states is maintained and how remote binding of the pentasaccharide confers its effect on the conformation of the reactive center loop.

EXPERIMENTAL PROCEDURES

Protein Expression and Derivatization—The S380C variant of antithrombin was produced on the recombinant β-glycoform antithrombin template of N135Q, as described previously (17), in order to reduce heparin binding heterogeneity. The variant was then reacted with 5-iodoacetamidofluorescein, obtained from Molecular Probes, Eugene, OR, on a 1-nl heparin-Sepharose column with a continuous loop run overnight at room temperature. After washing with low salt buffer (20 mM Tris, 10 mM sodium citrate, 5 mM EDTA, pH 7.4), the derivatized variant was eluted with a NaCl gradient as a single peak at about 1.7 M NaCl. The pooled fractions were 76% labeled, based on differential absorbance at 280 and 494 nm, and buffer-exchanged into 10 mM Tris, pH 7.4.

Crystalization—Crystallization was achieved as described previously (15, 21). Briefly, equimolar amounts of the derivatized or underderivatized variant and previously prepared plasma latent antithrombins were mixed with a concentrated solution of the precipitant in a 10-well glass plate. Final concentrations were 5 mg/ml protein, 10.5% PEG3400, 85 mM sodium cacodylate, pH 6.5. Crystals grew within 2 weeks of mixing and were yellow/green for the fluorescein-labeled variant.

Data Collection and Processing—Diffraction data were collected at the SRS Daresbury, UK, on beamline 7.2 for P14-fluorescein and 9.6 for the P14-Cys control using a single frozen crystal of each. Cryoprotection for the P14-fluorescein variant crystal was effected by quickly dipping the crystal into a solution of 10% methlypentanediol, 15% glycerol, 10.5% PEG 4000, 100 mM sodium cacodylate, pH 6.5, and quick freezing in the cryostream at 100 K. The P14-Cys, was dehydrated in 20% PEG 4000 and the cryoprotected with 20% glycerol. Useful data were obtained to a resolution limit of 2.85 and 2.8 Å for P14-fluorescein and P14-Cys, respectively, and processed using the programs of the CCP4 suite (22).

Molecular Replacement and Refinement—A cross-rotation function was co-computed using the program AMoRe (23) from the CCP4 package using the structure of the dimer of the β-isoglycoform of plasma antithrombin as the search model. Iterative rounds of rebuilding and refinement were conducted using the programs O (24) and Refmac (25) using the bulk solvent correction of CNS (35). Statistics of the final models are described in the Table I. Figures were made using Molscript (26), Bobscript (27), Grasp (28), and Raster3D (29, 30).

The central β-sheet A is in red; the reactive center loop is in yellow; helix D is in cyan; helix A is in green; and the P1 arginine in red space filling. The secondary structural changes in the pentasaccharide bound structure are in yellow, and the pentasaccharide is in a ball-and-stick representation. Glycosaminoglycan binding occurs via the carbohydrate bound structure are in yellow in the absence of glycosaminoglycan is reversed, with k2, and the reverse rate, k−2, is independent. Activation ultimately depends on release of constraints on the P1 Arg loop resulted from a mechanical force effected on strand 2A by the C-terminal elongation of helix D and that expulsion of the reactive center terminus of helix D and that expulsion of the reactive center loop resulted from a mechanical force effected on strand 2A by the C-terminal elongation of helix D. The structure of pentasaccharide-bound antithrombin revealed both helix D elongation and loop expulsion but not, as proposed, through direct interaction between the elongated portion of helix D and the pentasaccharide. This leaves the question of how pentasaccharide binding causes expulsion of the hinge region from β-sheet A. We hypothesize that the D-helix elongation is a consequence of the electrostatic effect of pentasaccharide binding and that expulsion is not effected by a mechanical force but rather a perturbation of surface electrostatics that help determine the equilibrium position between the native and activated states. This hypothesis is tested here by determining the structure of an antithrombin variant, N135Q, S380C, that is fully activated in solution in the absence of pentasaccharide due to the presence of a bulky fluorescein moiety bound at the P14 position (18). We specifically address the questions of how the solution equilibrium between the native and activated states is maintained and how remote binding of the pentasaccharide confers its effect on the conformation of the reactive center loop.

1 The abbreviations used are: PEG, polyethylene glycol; r.m.s., root mean square.

2 A. McCoy, X. Y. Pei, R. Skinner, L. Jin, and R. Carrell, unpublished structure.

3 A. McCoy, X. Y. Pei, R. Skinner, L. Jin, and R. Carrell, manuscript in preparation.

4 We refer to the α-glycoform of plasma antithrombin that contains all four N-linked carbohydrate chains as plasma antithrombin. Recombinant antithrombin variants S380C and S380C-fluorescein are made on the recombinant β-glycoform template of N135Q to eliminate glycosylation heterogeneity and increase affinity for heparin-Sepharose. Variants are designated P14-Cys and P14-fluorescein after the nomenclature of Schechter and Berger (1) where P1–P1′ is the scissile bond, and the P and P′ numbering increases towards the N and C termini, respectively.
This is most likely due to the difference in environment between solution and crystal and the constraints imposed by the formation of the dimer. The long and flexible linker allowed for the accommodation of the bulky fluorophore on the surface of β-sheet A with minimal perturbation of the backbone conformation for residues 378, 379, and 380 (P16 to P14). Fig. 2b is an electron density map of the region surrounding the fluorescein moiety and clearly demonstrates its position. The overall Cα electron density map of the region surrounding the fluorescein labeling cysteine is a part, and the heparin binding region. Fig. 2c is a ribbon diagram of P14-fluorescein illustrating the secondary structural changes in the heparin binding region, and Fig. 2d is a space filling representation of the fluorescein-labeled antithrombin variant colored according to amino acid r.m.s. deviation from plasma antithrombin. Residues with atoms deviating by more than 2 Å are colored magenta with the fluorescein in green and the reactive center Arg in red. Most of the atoms that deviated significantly were from side chains except for the N terminus of helix A and the C terminus of helix D which deviated significantly in main chain atoms.

### Hinge Region Interactions

Central to the question of what confers the special properties of antithrombin is the issue of why, in contrast to other serpins, the native conformation has its hinge region inserted in β-sheet A. The fully activated properties of P14-fluorescein antithrombin indicate that it exists predominantly in the loop-expelled conformation in solution. From this it is concluded that the fluorescein adduct is responsible for the change in position of the solution equilibrium. We therefore focused on the hinge region interactions that are perturbed by the fluorescein, and we identified a network of interactions in native antithrombin centered about its hinge region inserted in the α-helix. The fully activated properties of P14-fluorescein antithrombin indicate that it exists predominantly in the loop-expelled conformation in solution. From this it is concluded that the fluorescein adduct is responsible for the change in position of the solution equilibrium. We therefore focused on the hinge region interactions that are perturbed by the fluorescein, and we identified a network of interactions in native antithrombin centered about its hinge region inserted in β-sheet A.

### Table I

Data processing, refinement, and models

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<th>N135Q,S380C antithrombin</th>
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<td>1.488 (Daresbury SRS, station 7.2)</td>
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<td>Ala4-Lys29, Ile43-Val431</td>
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<td>NAG(β1,4)</td>
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<td>0.1% (Asn96 L)</td>
</tr>
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</table>

* $R_{merge} = \sqrt{\sum ||I_{hkl}|| - (\langle I_{hkl} \rangle)^2} / \sum I_{hkl}$.  
* NAG, N-Acetyl-D-glucosamine; Man, α-β-MANNOSE.  
* As described in column header.  
* The free set of reflections was selected to be the same as the free set used for the refinement of α-antithrombin and β-antithrombin.  
* Residues glycosylated and found in the same conformation in the latent and inhibitory forms of α-antithrombin.
FIG. 2. The structure of the activated S380C-fluorescein variant. a, a stereo representation of a Ca trace of the antithrombin dimer. The fluorescein variant is green, and the latent plasma antithrombin is in magenta with each 20th residue labeled and the fluorescein moiety in ball-and-stick. b, a sample of the electron density for the fluorescein moiety and surrounding residues. c, a ribbon diagram of the fluorescein variant in the same color scheme as Fig. 1 and the Cys380 sulfur in yellow space filling and the fluorescein in green space filling. The fluorescein on the surface of β-sheet A does not prohibit the partial loop insertion of the hinge region; however, helix A is extended by one turn and helix D by about one-half turn when compared with plasma antithrombin. d, a space filling representation of the P14-fluorescein structure colored according to atomic r.m.s. deviation. Amino acids containing atoms deviating by 2 Å or more from the structure of plasma antithrombin are colored in magenta, fluorescein in green, P1 arginine in red, and Lys222 in yellow (deviates by 1.9 Å). The orientation of the molecule is roughly as found in Fig. 1. The greatest difference between the two structures is in the region surrounding the fluorescein moiety and the heparin binding region.
Structural studies have revealed that the native state of antithrombin is stabilized by a network of interactions centered on Glu381 (P13) which interacts directly with s3A residue Tyr505 and helix F loop residue Arg197. Tyr505 also hydrogen-bonds to helix F loop residue Glu195, and Arg197 interacts via a salt bridge with s5A residue Glu374 and a stacking interaction with s5A residue Phe372. The basis of the solution activity of the fluorescein variant is the disruption of this network of interactions observed in native antithrombin. The greatest shift in side chain position is for Glu381 which is shifted by over 9 Å. Glu381 also plays a role in the stabilization of the pentasaccharide-activated conformation, c, by making interactions with Lys139 and Lys222 on strands 2A and 3A, respectively, upon closure of the five-stranded β-sheet A.

Disruption of these interactions could account for the loss of stability of the native P14-fluorescein variant (18) and, consequently, its fully activated solution properties. Comparison to the pentasaccharide-bound form also reveals the role of P13 Glu in stabilizing the activated, five-stranded form by shifting toward the heparin binding region and interacting with Lys139 and Lys222 on s2A and s3A, respectively. P13 Glu in the fluorescein variant shifts similarly due to the presence of the fluorescein group at P14 into close proximity to Lys139 but without making a hydrogen bond. The effect of the reorganization of the charged residues constituting the network surrounding P13 Glu is an alteration of the surface electrostatic potential of antithrombin (Fig. 4).

Structure of the Heparin Binding Domain—As Fig. 2c demonstrates, there is a change in secondary structure in the heparin binding region similar to that found in the pentasaccharide-bound antithrombin. The P14-fluorescein variant was made on the recombinant β template N135Q which prevents glycosylation at this position. We observed the presence of a hydrogen bond between Gln135 and a main chain N on s2A. To ensure that the altered helix D conformation for the P14-fluorescein variant was not due to the N135Q background, we also crystallized and solved the structure of the S380C,N135Q variant in the absence of fluorescein. As Fig. 5 demonstrates, the conformation of the recombinant control more closely resembles that of glycosylated plasma antithrombin with a surface-exposed side chain at position 135. This confirms that the structural changes in helix D of P14-fluorescein are the result of the presence of the fluorescein adduct in the hinge region.

Recent attention has been drawn to the potential role of helix A elongation in the mechanism of activation of antithrombin. Clearly, residues Arg46 and Arg47 are involved in the binding of heparin but are not critical (34). Surprisingly, our control structure of P14-Cys showed the same helix A elongation as the fluorescein-derivatized variant, extending to Asn45 as in the pentasaccharide-bound antithrombin. In addition, the structure of latent antithrombin in complex with the pentasaccharide shows both helix A elongation and helix P formation identical to the pentasaccharide-bound active monomer, but no elongation of helix D is observed (15). It therefore seems likely that helix A elongation and helix P formation facilitate tight interaction with the pentasaccharide but are not involved in the transmission of conformational change to the hinge region.

Fig. 2d demonstrates that, in addition to secondary structural changes in the heparin binding region, the presence of the fluorescein perturbs the conformation of the side chains on the surface of the variant. Interestingly, these limited changes form a link between the fluorescein moiety in the hinge region and the heparin binding region. The surface electrostatic potentials of the plasma and P14-fluorescein antithrombins oriented as in Fig. 2d are given in Fig. 4. Here too, the conformational link between the two regions is evident. More importantly, this comparison demonstrates that the effect of the perturbation of the interactions in the hinge region results in long range surface potential changes in the heparin binding region.

DISCUSSION

Regulation of antithrombin activity through binding to heparin-like oligosaccharides depends on a native fold which is kinetically inactive toward its target proteases. Therefore, how the native conformation is maintained and how heparin binding results in activation are central to understanding the special role antithrombin plays in the coagulation pathway. The structural studies of the P14-fluorescein variant of antithrombin described here directly addresses these issues. Interpretation of the structure in terms of the mechanism of activation requires a two-state model where the degree of activation depends on the position of the equilibrium between the two states, native and activated. The native state has a six-stranded β-sheet A due to the incorporation of the hinge region residues P15 and P14 as s4A, and the activated state has a five-stranded β-sheet A and resembles all of the other known structures of native serpins. Activation is normally achieved by the binding of a...
specific pentasaccharide sequence that stabilizes the five-stranded form resulting in its dominating the solution equilibrium. Due to the large structural changes accompanying pentasaccharide binding, the structures of the native and pentasaccharide-bound states are not alone sufficient for identification of the interactions that stabilize the native state in the absence of oligosaccharide. We crystallized the P14-fluorescein variant in a state that represents the left-hand side of the solution equilibrium illustrated in Fig. 1. This allows for the identification of the hinge region interactions involved in stabilizing the native conformation, since they would necessarily be altered by the presence of the fluorescein adduct.

We identified a network of electrostatic interactions centered on the P13 Glu which acts as a bridge between the overlying helix F loop, the hinge region, and flanking strands 3 and 5 of β-sheet A. These interactions are undone by the presence of the fluorescein in the structure of the variant. From the biochemical characterization of the variant, it is possible to conclude that disruption of this network alone is sufficient for the shift in equilibrium to favor the five-stranded activated state. The major conformational change caused by the presence of the fluorescein moiety was the shifting of the P13 Glu toward the heparin binding region. It is important to note that the P13 side chain could have moved elsewhere but was found oriented toward the heparin binding region in a position similar to that found in the pentasaccharide-bound structure. We believe that the observed change in side chain orientation of the P13 Glu represents a critical step in the activation mechanism first by 

The extent of activation upon glycosaminoglycan binding, at saturation, is related to the strength of binding defined by k1/k2 (Fig. 1). The extent of activation upon glycosaminoglycan binding, at saturation, is related to the strength of binding defined by k1/k2. 2) Glycosaminoglycan binding changes the surface electrostatic network that links the heparin-binding site and the reactive center loop so as to alter the interactions of P13 Glu that stabilize the loop-inserted conformation in the native state. 3) The pentasaccharide-induced change in surface electrostatics has two components. One is that pentasaccharide residues D, E, and F bind to helix D and increase k2 through charge neutralization and helix D elongation. The second is that the bound state is stabilized, decreasing k−2 through interactions between pentasaccharide residues G and H and Arg14 and Arg27 on the N terminus of helix A.

Efforts to create activated antithrombin variants for replacement therapy can now be focused on the interactions made by the P13 Glu residue. Destabilization of these interactions or stabilization of the activated conformer should result in a constitutively activated antithrombin.

REFERENCES

Structure of P14-Fluorescein Antithrombin