Articles

Mechanism of Heparin Activation of Antithrombin. Evidence for Reactive Center Loop Preinsertion with Expulsion upon Heparin Binding†

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ABSTRACT: A heparin-induced conformational change is required to convert antithrombin from a slow to a fast inhibitor of factor Xa. It has been proposed [van Boeckel et al. (1994) *Nat. Struct. Biol. 1*, 423−425] that the reactive center residue P14 is inserted into β-sheet A in native antithrombin and is displaced from the β-sheet by heparin binding, thereby altering the conformation of the reactive center and making it a better target for factor Xa binding. To test this hypothesis, we have characterized a P14 serine → tryptophan antithrombin variant. From changes in tryptophan fluorescence upon heparin binding, increased affinity for heparin, and partial activation of the variant against factor Xa, we conclude that the proposed mechanism of heparin activation is correct with respect to loop expulsion and that it may consequently be possible to create more highly activated antithrombin variants through suitable hinge region substitutions.

Antithrombin is a member of the serpin superfamily of serine proteinase inhibitors (Hunt & Dayhoff, 1980). Serpins inhibit proteinases by a branched pathway, suicide substrate inhibition mechanism (Rubin et al., 1990; Patston et al., 1991, 1994) (Figure 1) in which the intermediate, [EI′], that occurs at the branch point and which represents a normal species on the proteolytic cleavage pathway can be subverted from the cleavage pathway and become kinetically trapped as a covalent acyl-enzyme complex (Jörnvall et al., 1979; Jesty, 1979; Longas & Finlay, 1980; Lawrence et al., 1995; Wilczynska et al., 1995). For operation of the inhibitory pathway, the reactive center region must be able to efficiently insert into β-sheet A as an additional, central, strand. Since the rate-determining step in the serpin branched pathway mechanism precedes the branch point, the rate of reaction of a serpin with a given proteinase will be influenced by the conformation of the reactive center loop at the P1−P1′ bond and by flanking subsites of interaction with the proteinase. For many reactions of serpins with target proteinases the second-order rate constants for such reactions are relatively high (Table 1). A prominent exception is antithrombin, which inhibits its two principal targets, factor Xa and thrombin, at much lower rates. In the presence of heparin these rates of reaction are greatly increased to values comparable to those for other serpin−proteinase pairs (Table 1). For inhibition of thrombin most of the rate enhancement

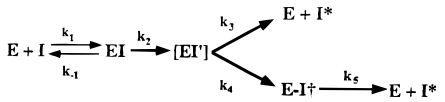

FIGURE 1: Branched pathway, suicide substrate inhibition mechanism of serpins, I, reacting with proteinase, E. The serpin initially forms a noncovalent, Michaelis-like complex, EI, which reacts further to give an intermediate, [EI′], which is on the normal substrate cleavage pathway. [EI′] can continue along this pathway with rate constant k_3 to give reactive center cleaved serpin, I*, and regenerated proteinase. An alternative pathway, with rate constant k_4, leads to a kinetically trapped covalent intermediate, E-I†, which decays only very slowly (hours to days) to cleaved serpin and free proteinase. Since steps leading up to formation of [EI′] are rate determining, changes in the relative values of k_4 and k_3 alter the *outcome* of the reaction without necessarily altering the rate of disappearance of serpin.

results from a bridging mechanism that effectively increases the rate of encounter of antithrombin and thrombin (Olson & Björk, 1991). However, for inhibition of factor Xa the rate enhancement results predominantly from a heparin-induced conformational change (Olson et al., 1992) that changes the conformation at the P1−P1′ scissile bond (Gettins et al., 1993). Heparin binds in a two-step process, with initial relatively weak binding through ionic interactions to the "low-affinity" native conformation, followed by a conformational change that increases the heparin affinity and gives the "high affinity" state of antithrombin (Olson & Shore, 1982).

X-ray structures of antithrombin (Schreuder et al., 1994; Carrell et al., 1994), of two other inhibitory serpins (Wei et al., 1994; Song et al., 1995), and of ovalbumin (Stein et al., 1991) have revealed two different types of structure in the reactive center region. In α_1-proteinase inhibitor (Song et al., 1995), the inhibitory variant of α_1-antichymotrypsin (Wei et al., 1994), and ovalbumin (Stein et al., 1991), the reactive center forms an α-helix or distorted helix attached to the

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Table 1: Rate Constants for Inhibition of Target Proteinases by Selected Serpins

serpin	second-order rate constant (M^{-1} s^{-1})	reference
α_1-proteinase inhibitor	HNE,[a] 6.5×10^7	Beatty et al., 1980
α_1-antichymotrypsin	cathepsin G, 5.1×10^7	Beatty et al., 1980
α_2-antiplasmin	plasmin, 3.8×10^7	Wiman & Collen, 1978
antithrombin		
−heparin	thrombin, 8.9×10^3; factor Xa, 2.5×10^3	Craig et al., 1989; Olson et al., 1991
+heparin	thrombin, 3.7×10^7; factor Xa, 1.3×10^6	
plasminogen activator inhibitor 1	u-PA, 1.1×10^7, t-PA, 2.3×10^7	Lawrence et al., 1990

[a] HNE, human neutrophil elastase.

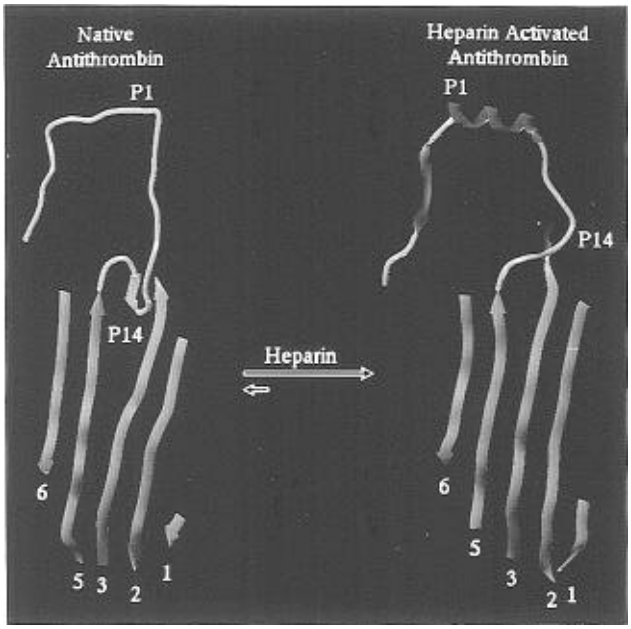

FIGURE 2: Schematic representation of the proposed structural changes in the reactive center region that result in heparin activation of antithrombin, based on X-ray-derived serpin structures. The polypeptide backbone is shown for β-sheet A (magenta) and the reactive center region, from its attachment point to strand 5 of β-sheet A to P10′. The "native" structure is that of the partially loop-inserted monomer from the heterodimer antithrombin structure of Schreuder et al. (1994). The structure of "heparin-activated" antithrombin is based on the structure of monomer A of native ovalbumin (Stein et al., 1991). The positions of the P14 and P1 residues are indicated. In the absence of heparin the loop-inserted structure is energetically favored, whereas heparin binding favors the loop-expelled structure. Note the large change in position and conformation of the P14−P1 reactive center residues between the two structures.

body of the serpin by two exposed stems. In both antithrombin structures, a very different conformation was observed in which partial insertion of the reactive center loop into β-sheet A had already occurred (Figure 2). The conformation of the remainder of the reactive center was consequently extended rather than helical. On the basis of these findings, a mechanism was proposed to explain how heparin binding results in a large increase in the rate of inhibition of factor Xa by antithrombin (van Boeckel et al., 1994). In this mechanism the normal conformation of antithrombin in solution is the partially loop-inserted one. The effect of heparin binding is to expel the sheet-inserted residues P15 and P14 from β-sheet A, so that the whole reactive center loop is exposed and is more like the other inhibitory serpins (Figure 2). Although this is a very attractive model, the antithrombin structures on which it is based are of antithrombin heterodimers in which only one molecule of the dimer adopts the partially loop-inserted conformation, whereas the other has the conformation of a latent or cleaved form of the serpin. Such dimers, in which only one monomer still retains inhibitory activity, cannot represent the normal situation in solution. It is therefore essential to examine the hinge region conformations of both native and heparin-bound antithrombins to determine whether the proposed mechanism of heparin activation is correct.

In this paper we have examined the properties of a P14 variant of antithrombin, in which the normal serine has been replaced by the fluorescent amino acid tryptophan. From the fluorescence of the P14 tryptophan both with and without heparin as well as the effects that this mutation has on heparin affinity, rate of inhibition of factor Xa, and stoichiometry of proteinase inhibition, we conclude that the normal solution conformation of antithrombin involves partial preinsertion of the reactive center loop, including residue P14, and that activation by heparin binding causes expulsion of this inserted region, as proposed by van Boeckel and colleagues.

METHODS

Isolation of S380W Antithrombin. The S380W[1] variant of antithrombin was created on a "wild-type" background of an N135Q mutation.[2] The latter change eliminates one glycosylation site and produces an antithrombin that resembles the naturally occurring plasma β-form (Peterson & Blackburn, 1985; Brennan et al., 1987). The advantage over true wild-type antithrombin as a background is reduction in carbohydrate heterogeneity and consequently heterogeneity of affinity for heparin (Fan et al., 1993; Turko et al., 1993). All references to control antithrombin refer to the same "high-affinity" glycoform (Turko et al., 1993) as was used for studies on the S380W variant. Site-directed mutagenesis was carried out on single-stranded M13mp19 containing the N135Q antithrombin cDNA template, as described previously (Turko et al., 1993). Introduction of the desired mutation was confirmed by sequencing. The mutated cDNA was excised by *Sal*I and *Xba*I, ligated into the expression vector pMAStop, and used to transfect BHK cells as previously described (Zettlmeissl et al., 1988). Serum-free cycles of medium were collected from roller bottles containing confluent stably transfected BHK cells. S380W antithrombin

[1] Abbreviations: S380W and N135Q, mutations in the antithrombin in which serine at position 380 and asparagine at position 135 have been changed to tryptophan and glutamine, respectively; SI, stoichiometry of inhibition; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel eleectrophoresis.

[2] As explained under Methods, the S380W antithrombin variant was created on a background of an N135Q mutation to reduce carbohydrate heterogeneity. Therefore "control" antithrombin always refers to the N135Q antithrombin, and the S380W variant refers to the S380W, N135Q double mutant antithrombin. Plasma antithrombin is always so named.

was purified on heparin−agarose, as described previously (Turko et al., 1993). S380W antithrombin was repurified by heparin−agarose chromatography immediately before each set of experiments to ensure full activity of the material. Purity was determined by nonreducing SDS−PAGE as described by Laemmli (1970). The extinction coefficient for S380W antithrombin was calculated to be $A^{1\%}_{280nm} = 7.5$ by adding the absorbance contribution of an average protein tryptophan (Gill & von Hippel, 1989) to the wild-type extinction coefficient of 6.5 (Nordenman et al., 1977). The effect of binding heparin was to decrease the extinction coefficient by only 1%. Since the results presented here indicate that the new tryptophan at position 380 changes its environment from buried to solvent exposed upon heparin binding, such a small change in extinction coefficient resulting from this environmental change confirms that the contribution of this tryptophan to the extinction coefficient is not very environmentally sensitive and that the calculated value of 7.5 is likely to be accurate.

Fluorescence Emission Spectra. Fluorescence emission spectra were collected on an SLM 8000 spectrofluorometer, with excitation at 280 nm and with band-passes of 4 and 1 nm for excitation and emission, respectively. A 10 s integration time was used for emission data collection between 300 and 400 nm with a step of 1 nm. A reference spectrum of $I = 0.3$ buffer was subtracted, and the resulting spectrum was smoothed. Protein concentrations were 1 μM in a final volume of 1.2 mL in 3 mL acrylic cuvettes. High-affinity heparin (M_r 9000) was added in a small aliquot to 13 μM. Four spectra were averaged for each condition.

Determination of SI. All SIs were determined by titration of proteinase with inhibitor, monitored spectrophotometrically by loss of proteinase activity, based on reaction of residual thrombin, trypsin, and factor Xa with the chromogenic substrates S-2238, S-2222, and Spectrozyme fXa, respectively. S-2238 and S-2222 were from Chromogenix Inc., and Spectrozyme fXa was from American Diagnostica Inc. Different ratios of S380W antithrombin and N135Q to proteinase were incubated at 25 °C, in a reaction volume of 20 μL, and allowed to react to completion. Residual proteinase activity was plotted against the ratio of inhibitor to proteinase, and the *x*-intercept of the linear regression fit was taken as the SI. All experiments were repeated at least twice. For thrombin and factor Xa reactions in the absence of heparin, polybrene was added to remove any possible heparin contamination. All reactions were carried out in an $I = 0.3$ buffer containing 20 mM NaP_i, 250 mM NaCl, 0.1 mM EDTA, and 0.1% PEG 8000, pH 7.4.

Determination of Rates of Inhibition. Second-order rate constants were determined at 25 °C for the reaction of N135Q and S380W antithrombin with thrombin, purified β-trypsin (Bock et al., 1989), factor Xa, and factor Xa with heparin under pseudo-first-order conditions in inhibitor and 10 nM proteinase, in the reaction buffer described in the previous section. Polybrene was added to those reactions conducted in the absence of heparin. The second-order rate constants (k_{app}) were calculated from observed pseudo-first-order rate constants (k_{obs}), obtained from the slope of the log of residual proteinase activity vs time, divided by the inhibitor concentration. Similar conditions were used for the reaction of N135Q and S380W antithrombin with factor Xa in the presence of heparin, except polybrene was added to the chromogenic substrate to ensure quenching of the heparin-dependent reaction. The observed pseudo-first-order rate constants were calculated for each heparin concentration from the slope of the log of residual proteinase activity vs time and plotted against heparin concentration to calculate the second-order rate constant for the uncatalyzed inhibition reaction, k_{uncat}, and the second-order rate constant for inhibition by the heparin−antithrombin complex, k_{cat}/K_m, from eq 1 (Olson et al., 1993), using the measured K_ds for

$$k_{obs} = \frac{k_{cat}}{K_m}[H]_0\left(\frac{[AT]_0}{[AT]_0 + K_d}\right) = k_{uncat}[AT]_0 \qquad (1)$$

the heparin−antithrombin interaction. Experiments were repeated at least twice, and obtained values were averaged. For calculation of the effective second-order rate constants, the value of k_{app} was multiplied by SI (Hood et al., 1994).

Determination of Heparin Dissociation Constants. Dissociation constants for the binding of heparin were determined as described previously (Olson et al., 1993) by following changes in fluorescence during the heparin titration. Measurements were made on an SLM 8000 spectrofluorometer exciting at 280 nm and monitoring at 340 nm, with a 4 nm excitation and 16 nm emission band-pass and with an integration time of 10 s. Stoichiometric titrations, carried out at 1 μM antithrombin, confirmed a 1:1 binding interaction, thereby corroborating the antithrombin concentration calculated from the extinction coefficient. Solutions (1.2 mL) of 50 nM N135Q and 25 nM S380W antithrombin were made in an $I = 0.6$ buffer containing 20 mM NaP_i, 550 mM NaCl, 0.1 mM EDTA, and 0.1% PEG 8000, pH 7.4, in acrylic cuvettes. The high ionic strength was necessary to weaken the binding sufficiently to allow accurate K_d measurements to be made. Five readings were averaged for each titration point, and titrations were repeated three times. After the reference titrations of buffer were subtracted, data were fit to eq 2 (Olson et al., 1993) by a

$$\Delta F = \Delta F_{max}\{P_0 + L_0 + K_d - [(P_0 + L_0 + K_d) - 4P_0L_0]^{1/2}\}/2P_0 \qquad (2)$$

nonlinear least squares method, as described previously, using the program Scientist for Windows by Micromath. ΔF is the observed fluorescence change, corrected for dilution and background, ΔF_{max} is the maximum fluorescence change, P_0 is the total antithrombin concentration, L_0 is the total heparin concentration, and K_d is the heparin−antithrombin dissociation constant.

CD Measurements. CD spectra were recorded on a Jasco 710 spectropolarimeter in a 1 mm path-length jacketed cell. Temperature was controlled by a Neslab water bath. Antithrombin concentrations were 0.25−0.5 mg/mL in $I = 0.15$ buffer (20 mM NaP_i, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4). All solutions were degassed. A bandwidth of 2 nm, step resolution of 0.5 °C, response time of 16 s, and rate of temperature change of 0.5 °C/min were used. Data are plotted as the first derivative of the ellipticity at 220 nm against temperature to make more clear the midpoint of the transition. Spectra were corrected for differences in concentration.

RESULTS AND DISCUSSION

The S380W Variant Is a Correctly Folded Antithrombin. A concern with any amino acid substitution is the possibility

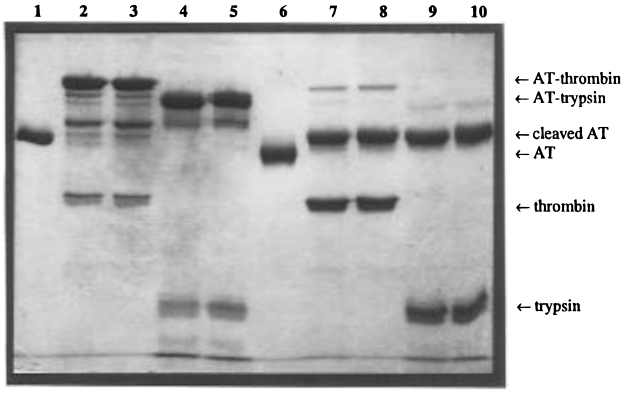

FIGURE 3: Ability of S380W variant antithrombin to form SDS-stable complexes with proteinase from the appearance of high MW bands on 10% SDS−PAGE under nonreducing conditions. The proportion of substrate-cleaved antithrombin was very much higher for the variant than for control antithrombin. Antithrombin (2 μg) was incubated with an equimolar amount of proteinase at 25 °C for 1 min in $I = 0.15$ buffer in the presence of heparin or for 15 min in the absence of heparin. The reaction was stopped by addition of PMSF, dilution with an equivalent volume of sample buffer, and boiling for 5 min. The stable antithrombin−proteinase complex ran as the highest molecular weight band, followed by the degraded complex, reactive center cleaved antithrombin, unreacted antithrombin, and free proteinase. Under nonreducing conditions, antithrombin that has been cleaved in the reactive center runs at higher apparent MW than uncleaved antithrombin. Lanes: 1−5, α-plasma antithrombin; 6−10, S380W antithrombin. Lanes: 1 and 6, antithrombin; 2 and 7, antithrombin incubated 1:1 with thrombin; 3 and 8, antithrombin incubated with thrombin in the presence of catalytic amounts of heparin; 4 and 9, antithrombin incubated 1:1 with trypsin; 5 and 10, antithrombin incubated 1:1 with trypsin in the presence of catalytic amounts of heparin.

of fundamentally altering the protein's structure and properties. To confirm that the serine → tryptophan substitution in antithrombin at P14 (residue 380) had not altered the normal folding of the protein, we examined the ability of the S380W variant to bind heparin, to be cleaved within the reactive center region, and to form SDS-stable covalent complexes with target proteinases. These properties are very sensitive to the correct folding of antithrombin and/or operation of the suicide inhibition mechanism. By these criteria,we judged the S380W variant to be a correctly folded antithrombin. Thus the S380W variant bound very tightly to heparin−agarose, eluting at 2.2 M NaCl, compared to 1.8 M NaCl for the control antithrombin. The variant was cleaved within the reactive center by both trypsin and thrombin, as indicated by a decrease in mobility on SDS−PAGE under nonreducing conditions (Figure 3) and an increase in mobility, from loss of the C-terminal 39 residues, when run under reducing conditions (not shown). The variant was simultaneously able to form SDS-stable complexes with thrombin and trypsin, shown by the appearance of higher molecular weight bands by SDS−PAGE (Figure 3), though the stoichiometries of inhibition (SI) were considerably higher for the variant than for the control, as shown by the low intensity of the band corresponding to the complex and the high intensity of cleaved antithrombin, arising from increased flux along the substrate branch of the pathway. The increased SI is not, however, an indication of misfolding or change in the *mechanism* of inhibition but rather of the relative kinetics of the two branches of the suicide inhibition pathway (see below).

P14 Is Preinserted and Displaced by Heparin Binding. To directly probe the environment of the P14 side chain and

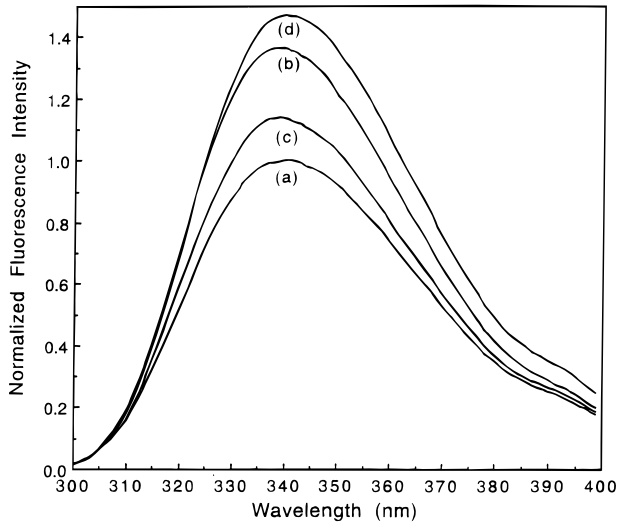

FIGURE 4: Tryptophan fluorescence emission spectra for the native and heparin-bound control and S380W antithrombins normalized to constant antithrombin concentration. Control antithrombin (a) underwent the expected fluorescence enhancement (36%) and 1 nm blue shift upon heparin binding (b). The presence of the additional tryptophan in S380W antithrombin resulted in a slightly blue-shifted spectrum for the native protein (c). Heparin binding to the variant produced a 29% enhancement and a 2 nm red shift (d).

changes in conformation upon heparin binding, we compared the fluorescence emission spectra of control and S380W variant antithrombins in the absence and presence of heparin. The spectra showed that the P14 tryptophan side chain in the S380W variant is in a hydrophobic environment in the absence of heparin and is changed to a much more polar environment upon heparin binding, consistent with initial burial of the side chain and subsequent expulsion upon heparin binding. Whereas the emission spectrum of the control antithrombin showed a 1 nm blue shift, accompanied by a 36% enhancement, that of the S380W variant showed a 2 nm red shift, accompanied by a 29% enhancement (Figure 4). The enhancement in the recombinant control antithrombin is the same as for the naturally occurring α-plasma antithrombin and arises from changes in the environment of one or two of the four tryptophans present in the wild-type protein (Olson & Shore, 1981). The lower percentage enhancement for the S380W variant corresponds to about the same absolute enhancement, since the initial fluorescence is increased by the presence of the additional tryptophan at P14. Fluorescence difference spectra between S380W and control antithrombins in the absence and presence of heparin showed more clearly the fluorescence properties of the P14 tryptophan alone (Figure 5). Heparin binding resulted in a dramatic 17 nm red shift from 337 to 354 nm, indicative of a large change in environment from hydrophobic to polar, as would be expected from expulsion of the residue from the β-sheet and consequent exposure of the previously buried tryptophan side chain.

S380W Antithrombin Has Increased Heparin Affinity. Elution of the S380W variant antithrombin from heparin−agarose at a higher salt concentration than for the control antithrombin (see above) indicated that the mutation had increased the affinity of the antithrombin variant for heparin. By determining the heparin dissociation constants for both the S380W variant and control antithrombins under the same conditions (Figure 6), we found that the affinity of the variant

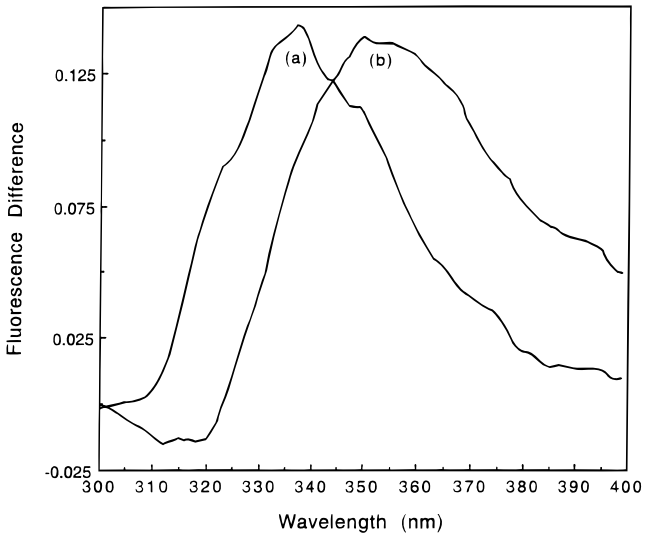

FIGURE 5: Tryptophan fluorescence emission difference spectra between S380W and control antithrombins to determine the properties of the additional P14 tryptophan. In each case the spectrum of control antithrombin, at the same protein concentration, was subtracted from that of the S380W variant. In the absence of heparin (a), λ_{max} was at 337 nm. In the presence of saturating high affinity heparin (b), λ_{max} shifted 17 nm to the red to 354 nm, indicating a change in environment of the P14 tryptophan from buried to solvent exposed.

was 7-fold greater than that of the control, with a change in K_d from 64 ± 17 to 9 ± 1 nM at $I = 0.6$, corresponding to a $\Delta\Delta G$ for binding of −5.5 kJ mol^{-1}. Such a perturbation in the energetics of heparin binding is understandable in terms of the model of heparin activation, since expulsion of the P14 side chain, as part of the heparin-induced conformational change, must contribute to the overall ΔG for heparin binding. If burial of the P14 serine in control antithrombin is energetically favorable, its expulsion upon heparin binding would contribute unfavorably to the energy of heparin binding. A reduction in favorable energy of side-chain burial for S380W antithrombin would then result in a less unfavorable contribution to the energy of heparin binding and consequently to an increase in heparin affinity. The observed changes therefore indicate that burial of the bulky indole ring into the constricted pocket available for the P14 residue side chain (Mourey et al., 1993; Schreuder et al., 1994; Carrell et al., 1994) is less favorable by about 5.5 kJ mol^{-1} than burial of the normal serine side chain. This implies that S380W antithrombin should be less stable than control antithrombin. Thermal denaturation studies of S380W and control antithrombins, using change in CD ellipticity at 220 nm, are consistent with this explanation, showing reduced stability for the variant, with a reduction in T_m from 60.7 °C for control to T_m of 55.2 °C for the S380W variant (Figure 7).

It has been suggested that part of the heparin activation mechanism in the reactive center region involves breaking a salt bridge between the P1 Arg393 and Glu255 (Carrell et al., 1994), thereby removing constraints on the peptide backbone in the vicinity of the scissile bond. However, we have previously found normal heparin affinity for a recombinant P1 Arg → Cys variant and for covalent derivatives involving attachment of large fluorescent reporter groups at P1 (Gettins et al., 1993). This implies that any involvement of P1 in the mechanics of the conformational change is smaller and much less direct than for the P14 residue.

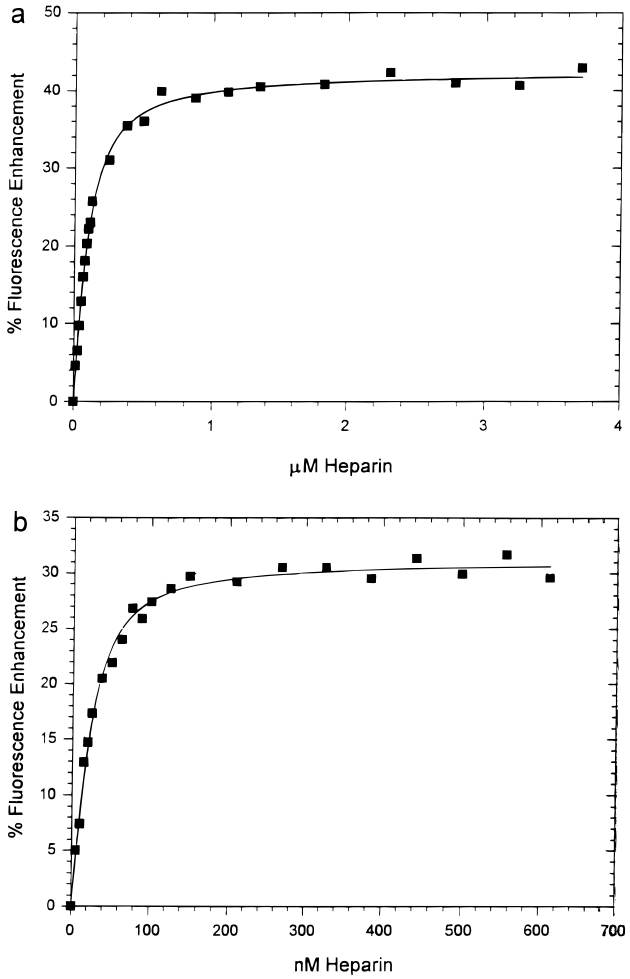

FIGURE 6: High-affinity heparin binding to antithrombins at $I = 0.6$ monitored by the change in tryptophan fluorescence as a function of added heparin. Panels: a, control antithrombin (50 nM); b, S380W variant antithrombin (25 nM). Solid lines represent least squares fits to the data obtained as described. Three titrations were performed for each antithrombin. The K_ds obtained were 64 ± 17 nM for control and 9.1 ± 1.3 nM for the S380W variant.

The S380W Variant Has Increased Substrate Reaction. To calculate rates of inhibition for serpins for which the substrate pathway is also important, it was first necessary to determine the SI value for each proteinase. As for other members of the serpin family that have charged or bulky residues substituted at position P14 (Davis et al., 1992; Hood et al., 1994; Lawrence et al., 1994), S380W antithrombin had greatly reduced efficiency as an inhibitor and thus greatly increased SIs. SIs for inhibition of factor Xa, thrombin, and trypsin, obtained from titrations of the variant with fixed concentrations of proteinase (Figure 8), were 29, 13, and 36, respectively (Table 2).

For factor Xa the SI was also determined in the presence of heparin, to permit calculation of the effective rate constant for inhibition for the S380W variant when complexed with heparin. The increase in SI, from 29 in the absence of heparin to 54 in its presence (Table 2), is understandable in terms of both the mechanism of proteinase inhibition by serpins and the proposed low and high-affinity states of antithrombin for heparin. The model of heparin binding (van Boeckel et al., 1994) is that the initial partially loop-inserted structure is the low-affinity state and the loop-expelled structure is the high-affinity state (Olson et al., 1981). Since inhibition of proteinase requires reinsertion of the reactive

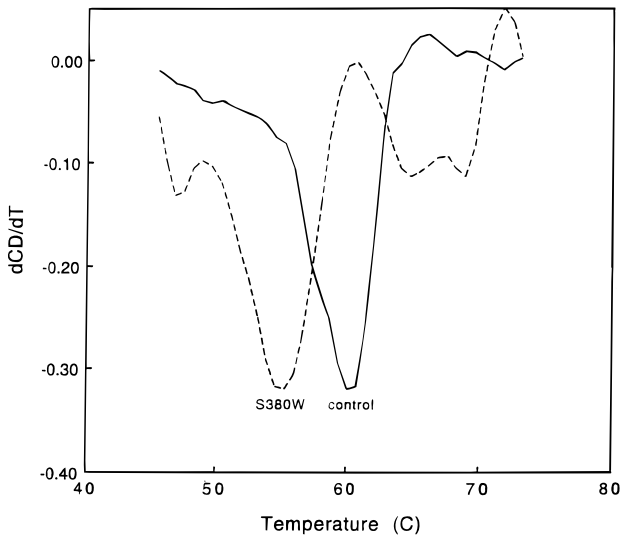

FIGURE 7: Thermal unfolding transition of control and S380W variant antithrombins monitored by change in CD ellipticity. The first derivative of the change in ellipticity at 220 nm as a function of temperature is shown for equivalent antithrombin concentrations. The T_m value was taken as the point of greatest rate of change (negative maximum) and was 55.2 °C for S380W antithrombin (dashed line) and 60.7 °C for control antithrombin (solid line).

center loop into β-sheet A, anything that makes such insertion more difficult, such as binding of heparin or an increase in affinity for heparin, will tend to increase SI. Such increases in SI have been seen for plasma antithrombin inhibition of both factor Xa (Olson et al., 1992) and thrombin (Olson, 1985; Olson et al., 1992).

S380W Antithrombin Has a Partially Activated Conformation. The ability of heparin to increase the rate at which antithrombin inhibits factor Xa can be understood in terms of antithrombin having a reactive center sequence that is well suited for factor Xa (P4−P1 = IAGR) (He et al., 1993; Stubbs et al., 1995) but that may not have the optimal conformation until heparin binds and causes a change in conformation for these residues (Figure 2). In contrast, antithrombin has a poor reactive center sequence for thrombin or other arginine-specific proteinases and consequently shows little sensitivity of the rate of inhibition of these proteinases to the conformation of the reactive center loop. We have previously shown that high- and low-affinity heparins, as well as dextran sulfate, cause different conformational changes in the antithrombin reactive center that correlate with different increases in rate of inhibition of factor Xa (Gettins et al., 1993). Mutations that change the conformation of the reactive center of antithrombin in the absence of heparin might therefore be expected to change the rate at which factor Xa is inhibited but not the rates at which trypsin or thrombin are inhibited. In particular, a mutation that partly mimics the heparin-activated conformation of antithrombin would be expected to have an increased rate of factor Xa inhibition. Since the indole side chain is much larger than that of serine, the position of the polypeptide backbone at the P14 Cα and adjacent residues must be further removed from the face of β-sheet A in the S380W variant than in control antithrombin, if the preinserted model of antithrombin applies. The S380W variant should therefore resemble a partially activated form of antithrombin. Using the SI values given above for inhibition of factor Xa, thrombin, and trypsin by S380W antithrombin, we found that the effective rates of inhibition of factor Xa (Figure 9) were

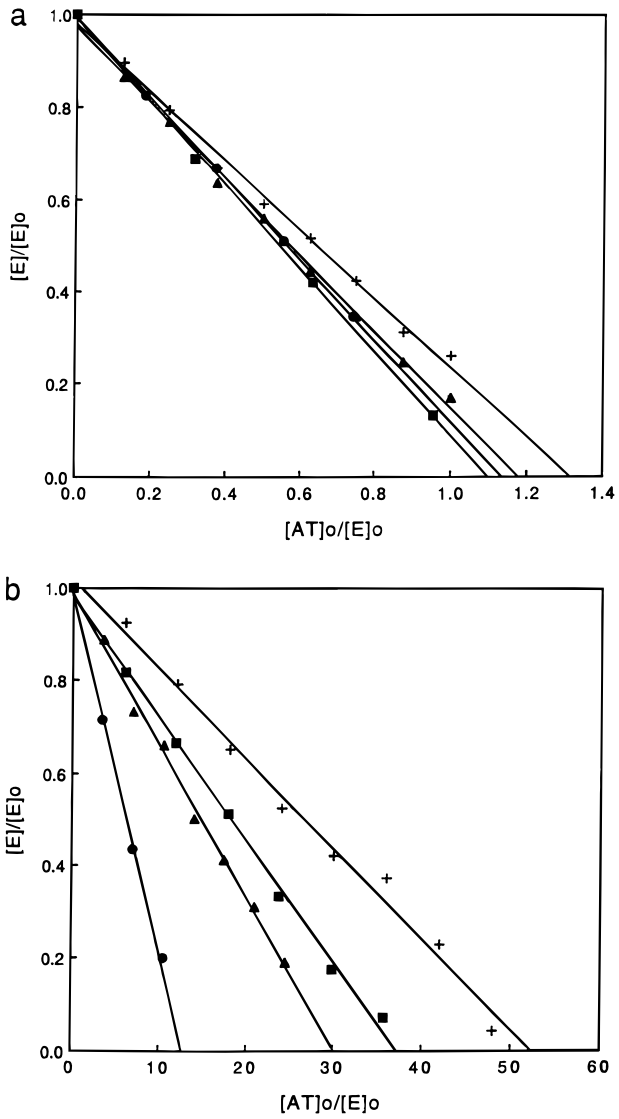

FIGURE 8: Determination of SI for control and S380W variant antithrombins for factor Xa ± heparin, trypsin, and thrombin from measurement of end-point residual enzymatic activity after incubation of different amounts of inhibitor with a fixed amount of proteinase. Panel a, control antithrombin, gave SI values close to 1 in all cases. Panel b, S380W antithrombin, gave SI ≫ 1 and with very different values for different proteinases. Symbols: circles, thrombin; squares, trypsin; triangles, factor Xa; crosses, factor Xa + heparin.

Table 2: Stoichiometries of Inhibition[a]

antithrombin	factor Xa	factor Xa + heparin	thrombin	trypsin
control	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
S380W	29 ± 2	54 ± 3	12.8 ± 0.1	36.0 ± 0.4

[a] Values are given as the mean of between two and six determinations, together with the range.

increased 2.6-fold over control antithrombin but were unaffected for inhibition of thrombin or trypsin (Table 3). Also as expected from this model, the effective rate of inhibition of factor Xa by the heparin−antithrombin complex was found to be similar for control and variant antithrombin (Table 3). This supports both the preinserted structure of native antithrombin and the proposed mechanism of heparin activation.

In light of these findings, we are now able to interpret previously unexplained observations on the rate of substrate

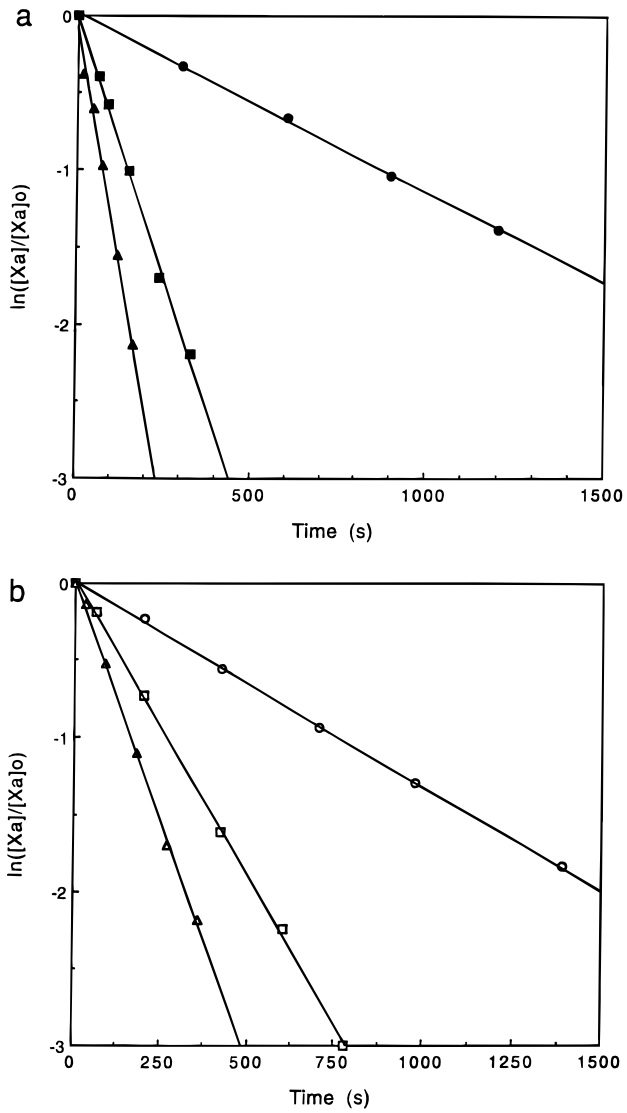

FIGURE 9: Determination of the rate constant for inhibition of factor Xa by control and S380W antithrombins in the presence and absence of heparin from semilog plots of residual enzymatic activity against time. Reactions were carried out under pseudo-first-order conditions. Panels: a, control antithrombin, without heparin (filled circles), 5 nM heparin (filled squares), and 10 nM heparin (filled triangles); b, S380W antithrombin, without heparin (open circles), 100 nM heparin (open squares), and 200 nM heparin (open triangles). Concentrations of antithrombin were 250 nM and 3 μM for the control and S380W variant, respectively. All reactions were 10 nM in factor Xa.

reaction of antithrombin−tetradecapeptide complexes with different proteinases. Such binary complexes with tetradecapeptides corresponding to reactive center residues P14−P1 have been made for antithrombin (Björk et al., 1992b) and α_1-proteinase inhibitor (Schulze et al., 1990). It appears that the peptide inserts into β-sheet A in the place that would normally be occupied by the reactive center upon formation of complex with proteinase. In consequence, the reactive center is no longer able to insert into β-sheet A, and the inhibitory pathway of the branched pathway mechanism is blocked. For serpins in which there is no preinsertion of part of the reactive center, formation of such binary complexes should not significantly alter the reactive center conformation and should therefore not significantly alter the rate of the substrate branch of the pathway. However, for antithrombin in which it is proposed that P15 and P14 are already inserted into β-sheet A, formation of a peptide binary

Table 3: Second-Order Rate Constants (M^{-1} s^{-1}) for Inhibition by Control and S380W Antithrombins[a]

antithrombin	factor Xa		factor Xa + heparin		thrombin		trypsin	
	k_{app}	k_{app}SI	k_{app}	k_{app}SI	k_{app}	k_{app}SI	k_{app}	k_{app}SI
control	$(4.6 \pm 0.4) \times 10^3$	$(5.6 \pm 0.4) \times 10^3$	$(1.10 \pm 0.02) \times 10^6$	$(1.43 \pm 0.02) \times 10^6$	$(6.1 \pm 0.3) \times 10^3$	$(6.7 \pm 0.3) \times 10^3$	$(1.88 \pm 0.1) \times 10^5$	$(2.06 \pm 0.1) \times 10^5$
S380W	508 ± 65	$(1.5 \pm 0.2) \times 10^4$	$(2.44 \pm 0.03) \times 10^4$	$(1.32 \pm 0.02) \times 10^6$	447 ± 25	$(5.7 \pm 0.3) \times 10^3$	$(6.1 \pm 0.3) \times 10^3$	$(2.2 \pm 0.1) \times 10^5$

[a] Values are given as the mean and range of two to six measurements.

complex should displace the preinserted reactive center, thereby resulting in a conformation that more closely resembles the heparin-bound conformation. Proteinases that are inhibited by antithrombin at rates that are increased by heparin in a conformationally dependent way should therefore react faster with such antithrombin−peptide complexes, whereas nonconformationally-dependent proteinases should react at normal rates. This is what has been observed. Factor Xa and plasmin both reacted much faster with antithrombin−peptide complexes (17-fold and 10-fold faster, respectively), whereas thrombin and factor IXa reacted at normal rates (Björk et al., 1992a).

Conclusions and Consequences. All of the results presented here for the structural or functional properties of the P14 S380W variant of antithrombin either directly or indirectly support the proposed mechanism of heparin activation of antithrombin (van Boeckel et al., 1994). In this mechanism residues P15 and P14 are already inserted into β-sheet A prior to heparin binding, representing the low heparin affinity state, and are expelled from the sheet in the second, conformational change, step of heparin binding to give the high-affinity state in which the reactive center loop is fully exposed and capable of adopting the most appropriate conformation for interaction with proteinases such as factor Xa and plasmin. The large red shift of the fluorescence emission spectrum of the P14 tryptophan upon binding heparin is as expected from displacement of the indole side chain from a buried to a solvent-exposed position. The 7-fold increase in heparin affinity of the variant results from easier displacement of the indole side chain, whereas the 2.6-fold increased basal rate of factor Xa inhibition (after correction for SI) reflects the somewhat changed conformation of the reactive center produced by the presence of the bulky tryptophan and represents a partially activated conformation. This raises the possibility that other changes in this critical hinge region of the reactive center might even more closely resemble the fully heparin-activated conformation and consequently give antithrombins that inhibit factor Xa at rates approaching those of antithrombin−heparin complexes. Thus residues whose insertion contributes adversely to the stability of the native structure would favor the loop-expelled conformation more highly. Of course, since loop reinsertion is required for subsequent inhibition, there may be a conflict between the requirement for favorable insertion for *efficient* inhibition and favorable displacement for optimal *rate* of inhibition.

Antithrombin appears to be a special case of a serpin in which an additional level of regulation of inhibition has been introduced by modifying the normal exposed and helical reactive center region so as to form the active conformation only in the presence of heparin. Heparin binding is required to expel the buried part of the loop so that the reactive center can adopt the conformation of other inhibitory serpins. Although such exposed conformations appear to be helical and therefore not obviously the correct initial conformation for interaction with target proteinase, they represent more conformationally flexible structures than the taught, extended loop conformation of antithrombin that is seen in the partially loop-inserted structure of antithrombin. It has been pointed out that the difference between good substrates and good lock-and-key protein proteinase inhibitors is the greater conformational lability of the former (Hubbard et al., 1991). Since inhibition by serpins requires a good substrate-like reaction, in contrast to the lock-and-key inhibition of Kunitz or Kazal inhibitors, the coiled and exposed reactive center loop may provide better flexibility than does a more extended but rigid conformation.

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