The Antithrombin P1 Residue Is Important for Target Proteinase Specificity but Not for Heparin Activation of the Serpin. Characterization of P1 Antithrombin Variants with Altered Proteinase Specificity but Normal Heparin Activation[†]

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Received December 26, 2000; Revised Manuscript Received March 28, 2001

ABSTRACT: Heparin has been proposed to conformationally activate the serpin, antithrombin, by making the reactive center loop P1 arginine residue accessible to proteinases. To evaluate this proposal, we determined the effect of mutating the P1 arginine on antithrombin's specificity for target and nontarget proteinases in both native and heparin-activated states of the serpin. As expected, mutation of the P1 arginine to tryptophan, histidine, leucine, and methionine converted the specificity of antithrombin from a trypsin inhibitor ($k_{assoc} = 2 \times 10^5\ M^{-1}\ s^{-1}$) to a chymotrypsin inhibitor ($k_{assoc} = 10^3-10^5\ M^{-1}\ s^{-1}$). However, heparin pentasaccharide activation increased the reactivity of the P1 variants with chymotrypsin or of the wild-type inhibitor with trypsin only 2−6-fold, implying that the P1 residue had similar accessibilities to these proteinases in native and activated states. Mutation of the P1 arginine greatly reduced k_{assoc} for antithrombin inhibition of thrombin and factor Xa from 40- to 5000-fold, but heparin normally accelerated the reactions of the variant antithrombins with these enzymes to make them reasonably efficient inhibitors ($k_{assoc} = 10^3-10^4\ M^{-1}\ s^{-1}$). Fluorescence difference spectra of wild-type and P1 tryptophan variant antithrombins showed that the P1 tryptophan exhibited fluorescence properties characteristic of a solvent-exposed residue which were insignificantly affected by heparin activation. Moreover, all P1 variant antithrombins bound heparin with ∼2−3-fold higher affinities than the wild type. These findings are consistent with the P1 mutations disrupting a P1 arginine−serpin body interaction which stabilizes the native low-heparin affinity conformation, but suggest that this interaction is of low energy and unlikely to limit the accessibility of the P1 residue. Together, these findings suggest that the P1 arginine residue is similarly accessible to proteinases in both native and heparin-activated states of the serpin and contributes similarly to the specificity of antithrombin for thrombin and factor Xa in the two serpin conformational states. Consequently, determinants other than the P1 residue are responsible for enhancing the specificity of antithrombin for the two proteinases when activated by heparin.

Antithrombin is the principal serpin family inhibitor of blood clotting proteinases. It inhibits these proteinases in a manner similar to that of other serpins by trapping the enzymes as stable acyl−intermediate complexes of a regular substrate reaction as a result of a massive serpin conformational change (*1*). However, it differs from other serpins in having multiple target proteinases with distinct substrate specificities and in requiring activation by heparin for inhibition of these proteinases at a physiologically significant rate (*1*, *2*). Antithrombin is activated by heparin as a result of the inhibitor binding to a specific pentasaccharide sequence in the polysaccharide (*3*). This binding induces structural changes in the serpin which both enhance its affinity for heparin and alter the conformation of a proteinase binding loop known as the reactive center loop (*4*, *5*). The pentasaccharide-induced conformational changes are sufficient to accelerate antithrombin inhibition of factor Xa, whereas an extended heparin chain capable of also binding the proteinase next to the bound serpin is additionally required to accelerate antithrombin inhibition of other proteinases (*4*, *6*, *7*).

As with most other serpins, a principal determinant of antithrombin specificity appears to be the P1 residue localized within the substrate recognition sequence of the reactive center loop (*8*). The importance of the P1 residue, arginine 393, in antithrombin is indicated from the observation that natural mutations of this residue appear to abolish antithrombin's inhibitory activity toward its target trypsin-like proteinases (*9*−*11*). In addition to determining specificity, the P1 residue has been proposed to play a critical role in heparin activation of antithrombin. According to this proposal, the P1 residue is inaccessible to proteinases in the native state due to the arginine side chain interacting with the serpin body and only becomes accessible through heparin inducing activating conformational changes in the reactive center loop (*12*). Support for this idea has come from the finding that natural mutations of the P1 residue enhance heparin binding

[†] This work was supported by National Institutes of Health Grants HL 39888 (to S.T.O.) and HL 30712 (to S.C.B.).

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10.1021/bi002933d CCC: $20.00 © 2001 American Chemical Society
Published on Web 05/10/2001

affinity, presumably by disrupting P1 arginine interactions which maintain the inhibitor in the native, low-heparin affinity state and, thereby, inducing the inhibitor into the activated, high-heparin affinity state (*11*). Additional evidence has come from the observation that the P1 arginine is unreactive with the enzyme peptidyl arginine deiminase in the native state but efficiently reacts in the heparin-activated state (*13*). Direct evidence that the P1 residue undergoes a structural change upon heparin activation has come from the finding that a fluorescent reporter group attached to a P1 Arg → Cys variant antithrombin exhibits a 100% fluorescence enhancement upon activation (*5*).

Contrasting these findings, an interaction of the P1 arginine with Glu 255 of the serpin body, observed in the crystal structure of native antithrombin and thought to render the P1 residue inaccessible in the native serpin, was found to not be disrupted in the structure of activated antithrombin complexed with heparin pentasaccharide (*12*, *14*, *15*). The P1 arginine interaction may thus be induced by the crystallization of antithrombin as a heterodimer wherein the reactive center loop of one active, loop-exposed antithrombin molecule interacts with the C β-sheet of a second inactive, loop-buried molecule and not exist in the structure of monomeric antithrombin. That the P1 residue is readily accessible to proteinases in the native unactivated state has been suggested by the efficient inhibition of trypsin by native antithrombin and the minimal activating effect of heparin on this inhibition (*16*). Moreover, differences in the accessibility of the antithrombin reactive center loop to proteinases do not appear to be an important factor in determining the large heparin enhancements in the rate of antithrombin inhibition of these proteinases, based on the finding that reactive center loop residues flanking the P1−P1′ scissle bond contribute similarly to the recognition of thrombin and factor Xa in both native and heparin-activated states of antithrombin (*17*).

To evaluate the contribution of the P1 residue to antithrombin's specificity for its target proteinases in comparison to that of other specificity determinants and assess how this contribution depends on heparin activation and conformational changes of the P1 residue, we have mutated the P1 arginine to tryptophan, histidine, leucine, and methionine. This was done to alter the P1 side chain specificity and thereby disrupt putative P1 arginine interactions with the serpin body and, in the case of tryptophan, also to provide a fluorescent reporter group for assessing conformational changes of the P1 residue which might be critical for the expression of P1 residue specificity. Our results confirm that while the P1 residue makes an important contribution to the specificity of antithrombin for thrombin and factor Xa, this contribution is similar in native and heparin-activated states and minimally affected by changes in P1 residue accessibility. Determinants other than P1 must thus be responsible for the enhanced specificity of the heparin-activated serpin for these enzymes such that efficient enzyme inhibition can still occur in the absence of the preferred P1 arginine side chain. Our findings additionally affirm previous evidence of a P1 arginine interaction with the serpin body in the native inhibitor, but they importantly establish this interaction to be of low energy and imply a mobile equilibrium between accessible and inaccessible states of the P1 arginine which minimally affects its reactivity.

EXPERIMENTAL PROCEDURES

Recombinant Antithrombins. Recombinant P1 antithrombin variants were constructed using N135Q or N135A antithrombin cDNA templates to eliminate glycosylation heterogeneity at the Asn 135 site and mimic the high-heparin affinity β-form of plasma antithrombin which is not glycosylated at this site (*18*−*20*). Site-directed mutagenesis was carried out by employing oligonucleotides containing the mutated codon and PCR to generate the mutant cDNA as previously described (*17*, *19*). All mutations were verified by DNA sequencing. For the R393W mutation, BHK cells were cotransfected with plasmids carrying either wild-type or mutant N135Q antithrombin cDNAs together with selection plasmids and stably transfected cell lines were selected by resistance to neomycin and methotrexate (*5*, *18*). Recombinant antithrombins were isolated from serum free cycles of roller bottle cultures of stably transfected BHK cells grown to confluence. The wild type or R393H, R393L, and R393M mutants were expressed in insect cells infected by baculovirus carrying wild-type or mutant N135A antithrombin cDNAs (*19*). Recombinant antithrombins were purified by heparin−agarose chromatography (*19*) and in the case of N135Q variants further purifed by DEAE-Sepharose and Sephacryl S-200 chromatography (*18*, *21*). Concentrations of recombinant antithrombins were determined from the absorbance at 280 nm using a molar absorption coefficient of 37 700 M^{-1} cm^{-1} (*22*) except for the P1 Trp variant, in which case the value for wild-type antithrombin adjusted for the presence of an additional tryptophan was used (*23*).

Proteinases and Heparins. Human α-thrombin was a gift from J. Fenton (New York State Department of Public Health, Albany, NY), and human α-factor Xa was obtained either by activation of purified factor X followed by purification on SBTI-agarose (*24*) or as a gift from P. Bock (Vanderbilt University, Nashville, TN). Active-site proteinase concentrations (>70% active for factor Xa and >90% active for thrombin) were determined on the basis of measured enzyme activities in a standard synthetic substrate assay using the calibrated relationship between activity and active-site concentration determined with several active-site-titrated enzyme preparations (*19*). Bovine α-chymotrypsin was from Worthington and used without purification. Bovine β-trypsin was purified from the commercial enzyme (Sigma type XIII) by SBTI-agarose chromatography (*25*). These enzymes were stored in 1 mM HCl and 10 mM $CaCl_2$ and their concentrations determined by active-site titration with fluorescein mono-*p*-guanidinobenzoate in the case of trypsin (*26*) or with *p*-nitrophenyl acetate in the case of chymotrypsin (*27*).

The synthetic heparin pentasaccharide corresponding to the binding sequence in heparin for antithrombin was a gift from M. Petitou (Sanofi Recherche, Toulouse, France). Full-length heparin chains of ∼26 saccharides (M_r ∼ 8000) containing the pentasaccharide were obtained by size and antithrombin affinity fractionation of commercial heparin (*4*, *21*). Concentrations of heparins were determined by stoichiometric titrations of antithrombin with the saccharides monitored by protein fluorescence changes (*4*, *21*).

Experimental Conditions. Most experiments were carried out at 25 or 37 °C as noted in 20 mM sodium phosphate buffers containing 0.1 ($I = 0.15$) or 0.25 ($I = 0.3$) M NaCl,

0.1 mM EDTA, and 0.1% polyethylene glycol 8000 (pH 7.4). Experiments involving trypsin or chymotrypsin were carried out in a 0.1 M Hepes buffer containing 0.1 M NaCl, 0.1% polyethylene glycol, and either 1 mM EDTA or 10 mM $CaCl_2$, at pH 7.4 ($I \sim 0.15$). Experiments at pH 6 and 8.5 were carried out in 50 mM MES, 50 mM Hepes buffers containing 0.1 mM EDTA, 0.1% polyethylene glycol, and either 0.58 M NaCl ($I = 0.6$) at pH 6.0 or 0.20 M NaCl ($I = 0.3$) at pH 8.5.

Stoichiometry and Kinetics of Antithrombin−Proteinase Reactions. Stoichiometries of antithrombin inhibition of proteinases in the absence and presence of heparin were determined in $I = 0.15$, pH 7.4 buffers at 25 °C as in past studies by titrating fixed concentrations of the enzymes (∼100 nM) with increasing concentrations of the inhibitor up to an ∼2:1 inhibitor:enzyme ratio and measuring residual enzyme activity after allowing complete reaction (*21*). Heparin, when present, was fixed at a concentration equimolar with the highest inhibitor concentration. Assays of chymotrypsin activity were carried out with 250 μM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma) as the substrate, trypsin activity with 100 μM S-2222 (Chromogenix), thrombin activity with 100 μM S-2238 (Chromogenix) or 50 μM tosyl-GPR-7-amido-4-methylcoumarin (Sigma), and factor Xa activity with 100−200 μM Spectrozyme FXa (American Diagnostica). Second-order rate constants for antithrombin association with proteinases in the absence and presence of heparin were determined at $I = 0.15$, pH 7.4, and 25 °C under pseudo-first-order conditions using a molar excess of inhibitor over enzyme that was at least 10 times the measured inhibition stoichiometry and pentasaccharide or full-length heparin concentrations ranging from substoichiometric to saturating (>98%) with respect to antithrombin (*20*, *21*). Enzyme inactivation was monitored at varying time intervals either by discontinuous assays of residual enzyme activity as in inhibition stoichiometry experiments or in the case of the P1 Trp variant reaction with chymotrypsin also by continuous assays of the decrease in enzyme activity in the presence of 250 μM succinyl-AAPF-*p*-nitroanilide substrate using 50−200 nM inhibitor and 3 nM chymotrypsin. Fitting of the decrease in enzyme activity by an exponential decay function with a zero end point in the case of discontinuous assays or the decrease in rate of substrate hydrolysis by an exponential function with a finite end point in the case of the continuous assay yielded the observed pseudo-first-order rate constant, k_{obs} (*21*). Second-order rate constants were obtained by linear regression analysis of the dependence of k_{obs} on the antithrombin concentration for uncatalyzed reactions. For heparin-catalyzed reactions, the dependence of k_{obs} on the heparin concentration was fit by the quadratic equilibrium binding equation to obtain k_{obs} at saturating heparin or the k_{obs} measured directly at saturating heparin (1.5−2-fold molar excess over antithrombin) and the second-order rate constant for the reaction of the antithrombin−heparin complex obtained by dividing by the antithrombin concentration (*4*, *28*). An exception was the full-length heparin catalyzed reaction of P1 Trp antithrombin with chymotrypsin, in which case the second-order rate constant was determined from the slope of the linear dependence of k_{obs} on heparin concentration at catalytic levels of heparin (*20*, *21*). In the case of the continuous assay of chymotrypsin inhibition, k_{obs} was corrected for substrate competition by multiplying by the factor $1 + [S]_o/K_M$, where $[S]_o$ is the substrate concentration and K_M the Michaelis constant, using a measured K_M value of 36 ± 2 μM.

Electrophoresis. SDS−PAGE analysis of antithrombin−proteinase reactions was carried out according to the method of Laemmli (*29*) in 10% gels under nonreducing conditions (*21*) by incubating in $I = 0.15$, pH 7.4 buffer at 25 °C a 2-fold molar excess of inhibitor (3−5 μM) with enzyme for 5−30 min except for uncatalyzed reactions of P1 antithrombin variants with thrombin and factor Xa in which case reaction times were extended to 1−3 days. Samples were quenched with a molar excess of AAPV, FFR, FPR, and EGR peptide chloromethyl ketone inhibitors for chymotrypsin, trypsin, thrombin, and factor Xa reactions, respectively, and then boiled in SDS sample buffer prior to electrophoresis (*28*).

Heparin Binding to Anithrombin. Heparin interactions with recombinant antithrombins were assessed by titrating fixed levels of antithrombin with heparin and monitoring the tryptophan fluorescence increase signaling heparin binding as in past studies (*4*, *20*, *21*) in $I = 0.15$, 0.3, 0.45, or 0.6 buffers at pH 6.0, 7.4, or 8.5 as noted. Excitation and emission wavelengths of 280 and 340 nm, respectively, were used and titration curves fit by the equilibrium binding equation to obtain the stoichiometry, K_D, and maximal fluorescence change (*21*). Qualitative assessments of heparin binding affinity were also made by measuring the salt concentration necessary to elute wild-type or variant antithrombins bound to a heparin−agarose matrix. Samples were loaded on a 5 mL Econo-Pac heparin−agarose column (Bio-Rad) equilibrated in $I = 0.15$ sodium phosphate buffer, and after the column had been washed with this buffer for 10 min (flow rate of 1 mL/min), a salt gradient to 3 M NaCl was applied over 30 or 35 min and the eluted antithrombin detected by protein fluorescence.

Fluorescence Spectroscopy. Protein fluorescence emission spectra of N135Q and R393W/N135Q antithrombins alone or in complex with full-length heparin were recorded in an SLM 8000 spectrofluorometer with excitation at 280 nm, and the emission wavelength was varied in 1−2 nm steps using 4 nm slits for both monochromators. Samples contained 0.1−0.5 μM antithrombin with or without equimolar heparin. Corrections were made for dilution and buffer background (<5%). Five to seven spectra from several experiments were averaged after normalizing the spectra from each experiment by dividing by the maximum fluorescence observed for wild-type antithrombin in the absence of heparin.

Kinetics of Dissociation of Antithrombin−Proteinase Complexes. Complexes of wild-type or R393W antithrombins with thrombin and factor Xa were prepared by incubating 5 μM antithrombin and 1 μM proteinase with 5 μM full-length heparin for 15 min in $I = 0.15$, pH 7.4 sodium phosphate buffer at 25 °C. Complexes were then extensively diluted to a final concentration of 1−5 nM into 400 μM S-2238 for thrombin complexes or 400 μM Spectrozyme FXa for factor Xa complexes at 37 °C (final pH of ∼7.3), and the acceleration of substrate hydrolysis due to the initial linear rate of appearance of active proteinase was monitored at 405 nm for 80 min (*28*, *30*). Data were fit by a parabolic function, and the dissociation rate constant was determined from linear plots of the initial rate of complex dissociation as a function of complex concentration using independently determined

Table 1: Association Rate Constants for Unaccelerated and Heparin-Accelerated Reactions of Wild-Type and P1 Variant Antithrombins with Proteinases at $I = 0.15$, pH 7.4, and 25 °C[a]

antithrombin	proteinase	k_{assoc} (M^{-1} s^{-1}) no heparin	with H5[b]	with H26[b]
wild type (N135Q)	chymotrypsin	~10	ND[c]	5.8×10^2
	trypsin[d]	1.4×10^5	4.0×10^5	9.0×10^5
	thrombin[d]	$(9.2 \pm 0.4) \times 10^3$	$(1.6 \pm 0.2) \times 10^4$	$(1.3 \pm 0.1) \times 10^7$
	factor Xa[d]	$(4.5 \pm 0.1) \times 10^3$	$(4.6 \pm 0.6) \times 10^5$	$(8.9 \pm 0.6) \times 10^5$
wild type (N135A)	thrombin[e]	$(9.4 \pm 0.4) \times 10^3$	$(1.1 \pm 0.1) \times 10^4$	$(9.0 \pm 0.5) \times 10^6$
	factor Xa[e]	$(4.8 \pm 0.2) \times 10^3$	$(6.1 \pm 0.2) \times 10^5$	$(1.2 \pm 0.1) \times 10^6$
R393W	chymotrypsin[f]	$(2.9 \pm 0.3) \times 10^5$	$(9.3 \pm 0.8) \times 10^5$	$(1.2 \pm 0.1) \times 10^7$
	trypsin	~2	ND	~9
	thrombin[g]	1.6 ± 0.1	1.9 ± 0.1	$(6.1 \pm 0.1) \times 10^3$
	factor Xa[g]	$(3.3 \pm 0.1) \times 10^1$	$(1.8 \pm 0.1) \times 10^3$	$(8.3 \pm 0.2) \times 10^3$
R393H[h]	chymotrypsin	$(5.4 \pm 0.7) \times 10^2$	$(3.4 \pm 0.1) \times 10^3$	$(7.6 \pm 0.5) \times 10^4$
	thrombin	$(1.9 \pm 0.3) \times 10^1$	$(2.6 \pm 0.1) \times 10^1$	$(2.2 \pm 0.1) \times 10^4$
	factor Xa	$(1.0 \pm 0.1) \times 10^2$	$(7.4 \pm 0.9) \times 10^3$	$(2.5 \pm 0.3) \times 10^4$
R393L[h]	chymotrypsin	$(8.1 \pm 0.4) \times 10^3$	2.6×10^4	$(5.9 \pm 0.8) \times 10^5$
	thrombin	1.5	ND	$(6.4 \pm 0.4) \times 10^2$
	factor Xa	2.2×10^1	$(2.1 \pm 0.2) \times 10^3$	$(1.0 \pm 0.1) \times 10^4$
R393M[h,i]	chymotrypsin	$\sim 1 \times 10^4$	ND	ND
	thrombin	ND	ND	4×10^3
	factor Xa	ND	ND	1×10^4

[a] Second-order association rate constants (k_{assoc}) for unaccelerated, pentasaccharide-accelerated, and full-length heparin-accelerated reactions of wild-type and variant antithrombins with proteinases were determined as described in Experimental Procedures. Errors represent the range or SE from two or more inhibition progress curves. [b] H5, heparin pentasaccharide; H26, full-length heparin with ~26 saccharides. [c] ND, not determined. [d] Values taken from ref *28*. [e] Values taken from refs *20* and *49*. [f] Values obtained from the data depicted in Figure 1. [g] Values obtained from the data depicted in Figures 3 and 4. [h] Values for heparin-accelerated reactions were obtained by saturating antithrombin with a 1.5−2-fold molar excess of heparin over antithrombin. [i] Only single progress curves were analyzed for each reaction due to the limited amounts of this variant.

turnover numbers for substrate hydrolysis by each enzyme to relate absorbance changes to changes in enzyme concentration (*28*).

RESULTS

Reactivity of P1 Antithrombin Variants with Chymotrypsin. P1 Trp antithrombin rapidly inhibited chymotrypsin, whereas inhibition of trypsin was barely detectable. Analysis of chymotrypsin inactivation progress curves under pseudo-first-order conditions as a function of the variant inhibitor concentration gave a second-order association rate constant (k_{assoc}) of $(2.9 \pm 0.3) \times 10^5$ M^{-1} s^{-1} for the reaction (Figure 1), comparable to the value previously reported for inhibition of chymotrypsin by a P1 Met → Trp variant of the α_1-proteinase inhibitor (*31*). Inhibition of trypsin by the P1 variant was estimated to be ~100000-fold slower. Substitution of the P1 residue with Leu, Met, or His similarly resulted in the appearance of inhibitory activity toward chymotrypsin, with second-order rate constants for chymotrypsin inhibition ranging from 10^3 to 10^4 M^{-1} s^{-1} (Table 1). By contrast, wild-type antithrombin inhibited trypsin efficiently ($k_{assoc} = 1.8 \times 10^5$ M^{-1} s^{-1}), in agreement with previous findings (*32*), but inhibited chymotrypsin ~10000-fold slower. Such results are consistent with the different substrate P1 residue preferences of chymotrypsin and trypsin and indicate that the mutations had altered the specificity of antithrombin from inhibiting trypsin-like to chymotrypsin-like enzymes.

Addition of saturating levels of pentasaccharide to the P1 Trp antithrombin variant increased the rate constant for chymotrypsin inhibition only ~3-fold, whereas a full-length heparin produced a ~40-fold increase in the inhibition rate constant (Figure 1). Comparable small effects of pentasaccharide and larger effects of full-length heparin on the rate of chymotrypsin inhibition were found with the P1 Leu and P1 His variants (Table 1). Saturation of the P1 Trp variant with full-length heparin only minimally enhanced the extremely slow rate of trypsin inhibition. Comparison with the wild-type antithrombin reaction with trypsin showed that the rate constant for trypsin inhibition was enhanced 3- and 7-fold by saturating levels of pentasaccharide and full-length heparins, respectively, the latter enhancement being in agreement with a previous report (*16*) (Table 1).

Titrations of chymotrypsin with P1 Trp antithrombin in the absence or presence of pentasaccharide or full-length heparins indicated inhibition stoichiometries of ~1 mol of inhibitor/mol of enzyme in all cases. SDS−PAGE confirmed the stoichiometric reaction of the P1 Trp variant with chymotrypsin to form an SDS-stable complex (Figure 2). Similar stoichiometries of chymotrypsin inhibition were found with other P1 variants after correction for the inactive inhibitor in the preparations as assessed from measured heparin binding stoichiometries (*20*).

Reactivity of P1 Variant Antithrombins with Thrombin and Factor Xa. In addition to the slow but detectable reactivity of the P1 Trp antithrombin variant with trypsin, the variant was also found to slowly inhibit both thrombin and factor Xa (Figure 4). That this inhibition was not due to contamination of the preparation with any wild-type inhibitor was suggested by the failure to observe any rapid trypsin inhibition by the variant serpin except at very high ratios of inhibitor to enzyme (50−500-fold). Under these latter conditions, a rapid reactivity of a fraction of the variant inhibitor preparation was detectable, whose extent and rate were consistent with contamination of the mutant with ~0.5% wild-type inhibitor. The minimal wild-type inhibitor contamination made no significant contribution to reactions conducted at lower inhibitor:enzyme ratios (10−30-fold) which were sufficient to satisfy the pseudo-first-order condition. The observed complete reaction of thrombin or factor Xa with the variant inhibitor in a single-exponential process

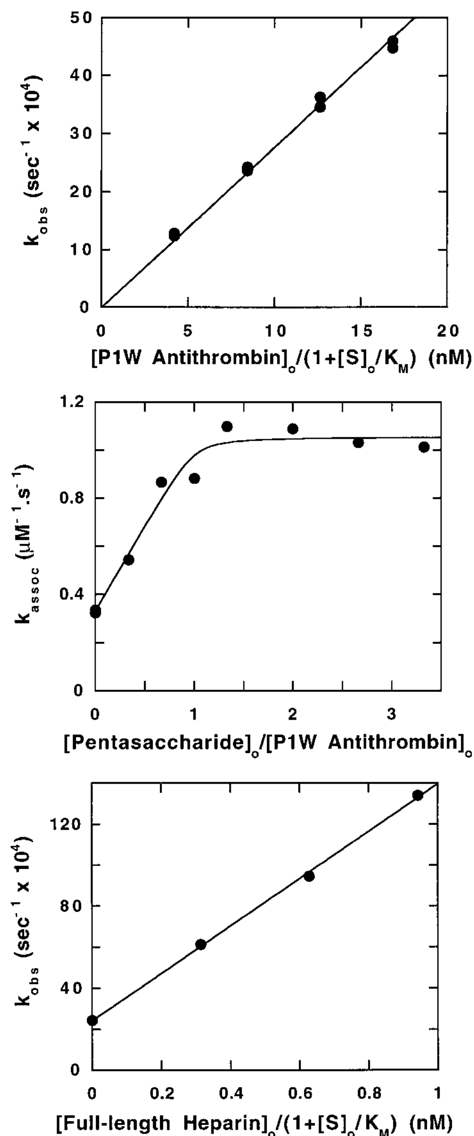


FIGURE 1: Kinetic analysis of reactions of unactivated and heparin-activated P1 Trp antithrombin with chymotrypsin. Shown are values of k_{obs} or the apparent k_{assoc}, calculated as the ratio of k_{obs} to the inhibitor concentration, for reactions of P1 Trp antithrombin with chymotrypsin plotted as a function of the inhibitor concentration or of the heparin concentration at a fixed inhibitor concentration of 100 nM. The top panel shows data for the unaccelerated reaction, the middle panel for the pentasaccharide-accelerated reaction, and the bottom panel for the full-length heparin-accelerated reaction. k_{obs} was measured by a continuous assay for enzyme inhibition employing a reporter chromogenic substrate as described in Experimental Procedures. The concentrations of inhibitor or heparin were divided by the factor $1 + [S]_o/K_M$ to correct for the competing effect of the substrate (S) on the inhibition reaction. Solid lines are fits of data by a linear function (top and bottom panels) or by a quadratic equilibrium binding function (middle panel). k_{assoc} was determined from the slopes of the linear plots or from the fitted end point of the nonlinear plot.

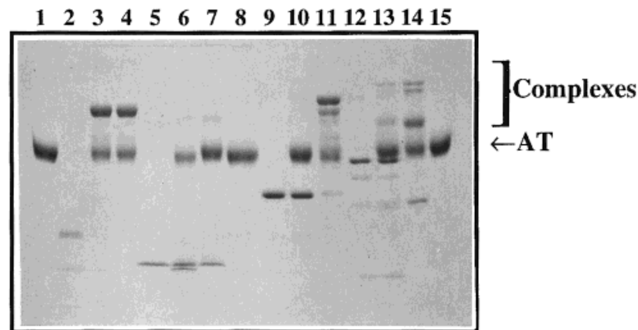


FIGURE 2: SDS−PAGE analysis of the reactions of P1 Trp antithrombin with proteinases. Shown are the products of the reactions of a 2−2.5-fold molar excess of P1 Trp antithrombin (5 μM) with chymotrypsin (2.5 μM) (lanes 3 and 4), trypsin (2.5 μM) (lanes 6 and 7), thrombin (2.0 μM) (lanes 10 and 11), and factor Xa (2.5 μM) (lanes 13 and 14). Reactions were carried out in the absence of heparin (lanes 3, 6, 10, and 13) or in the presence of full-length heparin equimolar with the inhibitor (lanes 4, 7, 11, and 14). Reaction times were 5 min for chymotrypsin and 30 min for trypsin, thrombin, and factor Xa. The unreacted proteins shown are P1 Trp antithrombin (lanes 1, 8, and 15), chymotrypsin (lane 2), trypsin (lane 5) thrombin (lane 9), and factor Xa (lane 12).

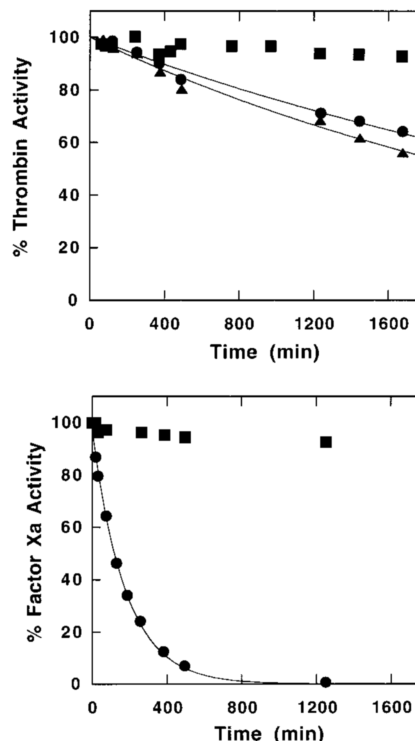


FIGURE 3: Progress curves for the inhibition of thrombin and factor Xa by P1 Trp antithrombin. Shown are the time-dependent decreases in enzyme activity resulting from the reactions of 5 μM P1 Trp antithrombin with 0.25 μM thrombin (top panel) or 0.25 μM factor Xa (bottom panel). Reactions were carried out in the absence of heparin (●) or in the presence of pentasaccharide equimolar with antithrombin (▲) as described in Experimental Procedures. Data for control incubations of enzyme alone are depicted as solid squares. Solid lines are fits by a single-exponential decay function with a zero end point.

at such ratios (Figure 3) thus indicated that the variant was responsible for the inhibition of these enzymes, a conclusion confirmed by SDS−PAGE analysis. Comparison of second-order rate constants for the P1 Trp and wild-type antithrombin reactions with either thrombin or factor Xa (Table 1) showed that the P1 mutation reduced thrombin reactivity by 5000-fold and factor Xa reactivity by ∼100-fold. Similar reduced reactivities of the P1 Leu variant were found with these enzymes, whereas the P1 His variant inhibited thrombin and factor Xa with 500- and 40-fold reductions in the inhibition rate constants, respectively (Table 1). Previous claims that a natural P1 His antithrombin had lost inhibitory activity toward thrombin are likely due to the testing of this activity for an insufficient period of time to detect the 500-fold reduced reactivity (*11*).

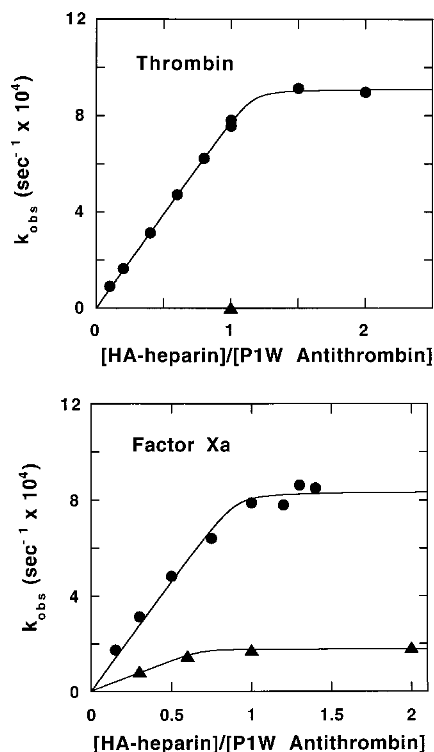


FIGURE 4: Effect of heparin on the reactions of P1 Trp antithrombin with thrombin and factor Xa. k_{obs} values for reactions of 100 nM P1 Trp antithrombin with 10 nM thrombin (top panel) or with 5 nM factor Xa (bottom panel) were measured from inhibition progress curves such as those in Figure 3 as a function of the molar ratio of pentasaccharide heparin (▲) or full-length heparin (●) to the P1 variant antithrombin as described in Experimental Procedures. Solid lines are fits by a quadratic binding function.

The full-length heparin greatly enhanced the ability of the P1 Trp antithrombin to inhibit thrombin as well as factor Xa. The observed pseudo-first-order rate constant for the inhibition of these enzymes showed a saturable dependence on the heparin concentration which plateaued at a molar ratio of heparin to inhibitor of ~1 (Figure 4), indicating heparin enhancements of the second-order rate constants for these reactions of ~3000- and ~300-fold, respectively, similar to the heparin enhancements of the wild-type inhibitor reactions with these enzymes (Table 1). Pentasaccharide heparin similarly enhanced the rate of P1 Trp antithrombin inhibition of factor Xa, but a somewhat lower second-order rate constant at inhibitor saturation representing a ~70-fold rate enhancement was found in this case (Figure 4 and Table 1). By contrast, the pentasaccharide only slightly stimulated the variant inhibitor to inactivate thrombin, similar to the <2-fold stimulation of the wild-type inhibitor−thrombin reaction by pentasaccharide (Figure 3 and Table 1). Similar heparin rate-enhancing effects on the reactions of P1 Leu, P1 His, and P1 Met antithrombin variants with these enyzmes were observed (Table 1).

Titrations of thrombin or factor Xa with the P1 antithrombin variants could only be carried out in the presence of heparin. P1 Trp antithrombin inhibited factor Xa with stoichiometries slightly greater than 1 mol of inhibitor/mol of enzyme when complexed with either pentasaccharide or full-length heparins, whereas it inhibited thrombin with a stoichiometry of ~1 when bound to pentasaccharide and with a value of 2.7 ± 0.1 mol of inhibitor/mol of enzyme when bound to the full-length heparin. Similar inhibition stoichiometries were found for the heparin-accelerated reactions of other P1 variants with these enzymes. This behavior paralleled that of heparin-catalyzed wild-type antithrombin reactions with the enzymes. The stoichiometry of ~2−3 observed for the thrombin reaction in the presence of the full-length heparin arises from heparin stimulating a substrate mode of reaction of antithrombin with thrombin (*4*, *6*). SDS−PAGE analysis confirmed that the P1 Trp antithrombin formed complexes with thrombin and factor Xa in the presence of heparin over short reaction times (Figure 2) and also with these enzymes in the absence of heparin over longer reaction times (not shown). SDS-stable complexes were also observed to be formed in reactions of P1 Leu and P1 His variants with thrombin.

Table 2: Rate Constants for Dissociation of Antithrombin−Proteinase Complexes at $I = 0.15$, pH 7.3, and 37 °C[a]

antithrombin	proteinase	k_{diss} (s^{-1})
wild type	thrombin	$(6.4 \pm 0.6) \times 10^{-7}$
	factor Xa	$(5.4 \pm 0.5) \times 10^{-7}$
R393W	thrombin	$(6.3 \pm 0.1) \times 10^{-7}$
	factor Xa	$(6.5 \pm 0.1) \times 10^{-7}$

[a] k_{diss} values ± standard error were obtained from linear regression analyses of the dependence of the initial rates of complex dissociation on the concentration of complex for four or five complex concentrations as described in Experimental Procedures.

Analysis of the kinetics of dissociation of wild-type and P1 Trp variant antithrombin complexes with thrombin and factor Xa gave dissociation rate constants for the variant inhibitor complexes similar to those of the wild-type inhibitor complexes despite the enormous decreases in association rate constants (Table 2). These findings indicated that while the rate of formation of the complexes was critically dependent on the P1 residue, the stability of these complexes was independent of the nature of the P1 residue.

Heparin Binding to and Conformational Activation of the Variant Antithrombin. Titrations of tryptophan fluorescence changes in antithrombin induced by heparin binding at $I =$ 0.15 and 100 nM inhibitor showed that the full-length heparin bound stoichiometrically to wild-type and P1 variant antithrombins due to the high heparin affinity under these conditions (estimated K_Ds of ~1 nM) (*4*, *20*). Titrations at higher ionic strengths ($I = 0.3$ or 0.45) were carried out to weaken the binding affinity and allow a more precise determination of any differences in heparin affinity between wild-type and variant antithrombins. Such titrations revealed a 2−3-fold tighter binding of heparin to the P1 variant antithrombins than to the wild-type inhibitor, and this higher heparin affinity was seen for both pentasaccharide and full-length heparins in the case of the P1 His variant (Table 3). Analytical chromatography of wild-type and P1 variant antithrombins on a heparin−agarose column showed that the variant antithrombins eluted at a salt concentration higher than that required to elute the wild-type inhibitor (Table 3), confirming that all P1 mutations produced similar increases in heparin affinity. Relative fluorescence enhancements induced in antithrombin by heparin at saturation were normal for the P1 His and Leu variants but reduced for the P1 Trp variant due to the additional tryptophan producing a higher initial fluorescence. When the enhancement of the latter variant was expressed relative to the initial wild-type inhibitor fluorescence, it was indistinguishable from that of the wild

Table 3: Dissociation Constants, Stoichiometries, and Fluorescence Enhancements for Heparin Binding to Variant Antithrombins at 25 °C and pH 7.4[a] and Salt Concentrations at Which Antithrombins Elute from a Heparin−Agarose Column[b]

antithrombin variant	I	K_D (nM)	stoichiometry	$\Delta F_{max}/F_o$	[NaCl] (M)
N135Q	0.3	7.9 ± 1.2	0.97 ± 0.09	0.37 ± 0.03	1.55
R393W/N135Q	0.3	2.5 ± 0.3	0.91 ± 0.02	0.27 ± 0.01	1.84
N135A	0.3	5.6 ± 0.7	0.82 ± 0.03	0.38 ± 0.05	1.87 ± 0.02
R393H/N135A	0.3	2.8 ± 0.6	0.8 ± 0.1	0.33 ± 0.02	2.02 ± 0.03
R393L/N135A	0.3	4.0 ± 0.5	0.62 ± 0.04	0.35 ± 0.02	2.08 ± 0.01
R393M/N135A	–	ND[c]	ND	ND	2.13 ± 0.01
N135Q	0.45	78 ± 7	ND	0.50 ± 0.01	–
R393W/N135Q	0.45	44 ± 4	ND	0.33 ± 0.02	–

[a] Binding parameters from at least two titrations of antithrombin with full-length heparin are given with errors representing either the range or standard error depending on the number of determinations. [b] NaCl concentrations ± the range or standard error from at least two experiments.[c] ND, not determined.

Table 4: Effect of pH on Dissociation Constants for Heparin Binding to N135A and R393H/N135A Antithrombins at 25 °C

pH	I	heparin form[b]	K_D (nM)[a] N135A	K_D (nM)[a] R393H/N135A	$K_{D,N135A}/K_{D,R393H/N135A}$
6.0	0.6[c]	H26	32 ± 1 (2)	21 ± 4 (4)	1.5
7.4	0.3	H26	5.6 ± 0.7 (4)	2.8 ± 0.6 (5)	2.0
7.4	0.3	H5	26 ± 8 (4)	10 ± 1 (3)	2.6
8.5	0.3	H26	43 ± 2 (3)	17 ± 1 (2)	2.5

[a] Average K_D values are given ± the range or standard error with the number of titrations indicated in parentheses. [b] H5, heparin pentasaccharide; H26, full-length heparin with ~26 saccharides. [c] Titrations at pH 6 were carried out at a higher ionic strength to weaken the affinity and improve the precision of the measurements.

type, suggesting that the fluorescence of the P1 tryptophan was not sensitive to conformational activation.

To determine whether conversion of the P1 His from a neutral to a positively charged side chain would cause the heparin affinity of the variant to revert back to the wild-type affinity as previously reported (*11*), the pH dependence of heparin binding to P1 His and wild-type antithrombins was determined. Table 4 shows that while a higher heparin affinity of the P1 His variant was maintained over the pH range of 6−8.5, a trend from an ~2.5-fold higher affinity at pH 8.5 where the His side chain should be neutral to an ~1.5-fold higher affinity at pH 6 where the His side chain should be mostly positively charged was observed.

Fluorescence Emission Spectra of Wild-Type and Variant Antithrombins in Native and Activated Conformations. Figure 5A compares the fluorescence emission spectra of wild-type and P1 Trp antithrombins in the absence and presence of saturating levels of full-length heparin (93−97%). The P1 Trp variant exhibited a higher fluorescence intensity and small red shift (~2 nm) of the spectrum relative to that of the wild-type inhibitor, consistent with the presence of the additional surface tryptophan residue. Heparin produced similar increases in the fluorescence intensity of both wild-type and mutant inhibitors as indicated by the indistinguishable difference spectra of complexed and free serpin (Figure 5C), in keeping with the results of the fluorescence titrations at a fixed wavelength. To determine the spectrum of the P1 tryptophan residue in the absence and presence of heparin activation, the wild-type inhibitor spectrum was subtracted from the mutant inhibitor spectrum for both the free and heparin-complexed proteins. Similar difference spectra with approximately the same emission maxima and intensities were obtained (Figure 5B). The P1 tryptophan accounted for ~15% of the total fluorescence of the unactivated inhibitor. Moreover, its emission maximum of ~350 nm was 10 nm red-shifted relative to the emission maximum of the wild-type serpin and approached that of *N*-acetyltryptophan amide (λ_{max} ~ 363 nm) under the same conditions, consistent with the tryptophan being largely solvent exposed.

DISCUSSION

These studies were undertaken to evaluate the relative importance of the P1 residue of antithrombin in determining the specificity of the serpin for the target proteinases, thrombin, and factor Xa, in comparison with other determinants of inhibitor specificity, and to test whether the P1 residue contribution to specificity depended on heparin activation of antithrombin. In particular, we were interested in evaluating the proposal that the P1 arginine residue of antithrombin is a crucial determinant of the enhanced inhibitory activity of the serpin when it is activated by heparin (*11*−*13*, *32*). While the findings of the present study support an important role for the P1 residue in determining antithrombin proteinase specificity, they do not support the proposal that the P1 residue acts as a key mediator of the enhanced proteinase reactivity of the heparin-activated serpin by undergoing a large change in accessibility as a result of activation. Such a change in P1 residue accessibility was anticipated on the basis of the increased level of exposure of the loop upon heparin activation resulting from the loop being partially buried in β-sheet A in the native conformation and expelled from the sheet in the activated state (*12*, *14*, *15*, *48*) and on evidence of an altered environment and reactivity of the P1 residue upon heparin activation (*5*, *13*). However, our present results have shown that while mutations of the P1 arginine to large hydrophobic residues switch the specificity of antithrombin from a trypsin to a chymotrypsin inhibitor, the reactivities of the wild-type inhibitor with trypsin and of the P1 variant inhibitors with chymotrypsin are enhanced only 2−6-fold due to conformational activation by the heparin pentasaccharide. The accessibility of the P1 residue of wild-type or variant antithrombins to proteinases thus cannot be greatly affected by conformational changes in the loop induced by heparin activation. The previous observation that the P1 arginine was unreactive with peptidyl arginine deiminase in the native conformation but efficiently reacted with the enzyme in the heparin-activated conformation (*12*, *13*) may therefore reflect unfavorable steric or charge properties of the deiminase which hinder its access to the P1 arginine in the native loop-constrained conformation

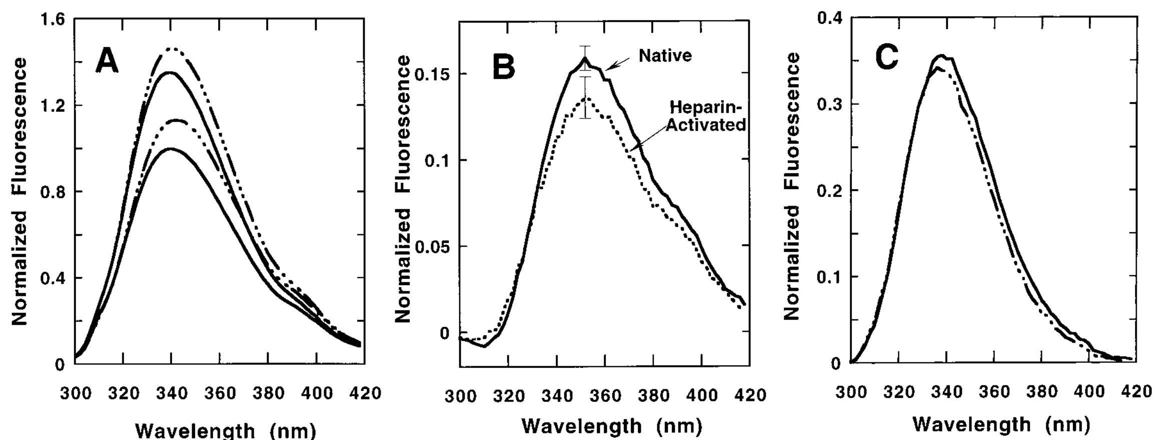


FIGURE 5: Fluorescence difference spectra analysis of the effect of heparin on the P1 Trp emission spectrum. Average fluorescence emission spectra of wild-type (—) and P1 Trp variant (—•••—) antithrombins (100−500 nM) are shown in the absence and presence of equimolar full-length heparin from seven experiments (A). All fluorescence values are expressed relative to the peak fluorescence of wild-type antithrombin. The difference between P1 Trp variant and wild-type antithrombin spectra in the absence (—) and presence (•••) of heparin yielded the average spectrum ± standard error of the P1 Trp residue in native and heparin-activated states (B). The difference between wild-type antithrombin with and without heparin (—) or between the variant antithrombin with and without heparin (—•••—) reflects the average spectrum of the fluorescence enhancement induced by heparin (C).

since trypsin does not show any such hindered access. Both the wild-type antithrombin reaction with trypsin and variant inhibitor reactions with chymotrypsin were accelerated more substantially by a full-length heparin, but such larger rate enhancements likely reflect the contribution of heparin bridging antithrombin and proteinase and are thus not expected to involve the P1 residue (*4*, *6*). This conclusion is supported by the correlation between the extent of the additional rate enhancements and the number of conserved basic residues in trypsin and chymotrypsin in the region corresponding to the heparin binding exosites of thrombin and factor Xa which mediate heparin bridging (*33*−*35*); i.e., one basic residue in the exosite, Lys 169, is conserved in trypsin, whereas three such residues, Lys 93, Lys 169, and Lys 175, are conserved in chymotrypsin.

The observation that mutation of the P1 arginine of antithrombin reduced inhibitor reactivity with trypsin much more than it did with thrombin and factor Xa is consistent with specificity determinants other than the P1 residue contributing to the reactivity of antithrombin with the clotting enzymes. The additional finding that this reactivity was accelerated by heparin to an extent comparable to that of the wild-type inhibitor reactions with these enzymes indicates that the heparin accelerating mechanism is largely unaltered by the P1 mutations despite the P1 specificity mismatch. This confirms that the P1 residue contributes similarly to antithrombin specificity in native and heparin-activated states of the serpin, and thus, differences in P1 residue accessibility in these states do not significantly contribute to the heparin activating mechanism. It follows that new specificity determinants other than the P1 residue must become available in the antithrombin−heparin complex to enhance recognition of the serpin by thrombin and factor Xa. As we have recently shown (*17*), such determinants do not appear to involve the reactive center loop P6−P3′ region based on the finding that mutations in this region do not significantly affect heparin enhancement of the rates of antithrombin inhibition of thrombin or of factor Xa. The origins of the new specificity determinants depend on the target enzyme and appear to involve, in the case of thrombin, a bridging site on heparin adjacent to the pentasaccharide site for antithrombin and, in the case of factor Xa, an unidentified exosite on antithrombin (*17*). A bridging-site determinant on heparin also contributes to the enhanced recognition of heparin-activated antithrombin by factor Xa in the presence of physiologic levels of calcium since the heparin binding exosite of this enzyme only becomes accessible through calcium binding to its γ-carboxyglutamic acid domain (*36*, *37*).

The contribution of the P1 residue to antithrombin specificity can be estimated from the ratio of the association rate constants for mutant and wild-type inhibitor reactions to minimally range from 2000- to 10000-fold for thrombin and from 100- to 300-fold for factor Xa for P1 Trp, Leu, and Met substitutions. The smaller reductions in specificity due to the P1 His replacement of 400−500-fold for thrombin and 40−80-fold for factor Xa may reflect perturbations of the His pK_a when it is bound in the S1 site of the proteinase which result in protonation and enhanced binding of the positively charged histidine side chain. The greater losses in antithrombin specificity for thrombin than for factor Xa due to the P1 arginine mutations imply that the more hydrophobic P1 side chains are accommodated better in the S1 site of factor Xa than in the S1 site of thrombin. It is not a surpise that residues other than arginine can be accommodated in the largely hydrophobic S1 sites of thrombin and factor Xa. Heparin cofactor II and a variant fibrinogen both interact with thrombin despite having P1 leucine (*38*, *39*) and histidine residues (*40*), respectively, due to their interaction with a common exosite in thrombin (*41*). Similarly, factor Xa binds the inhibitor, tick anticoagulant peptide, with high affinity despite having a P1 tyrosine residue, due to interaction of the inhibitor with a factor Xa-specific exosite (*42*, *43*). Interactions of nonreactive center loop determinants of heparin-bound antithrombin with exosites on thrombin and factor Xa can thus similarly account for the ability of both wild-type and P1 variant antithrombins to show an efficient reactivity with these enzymes in the presence of heparin.

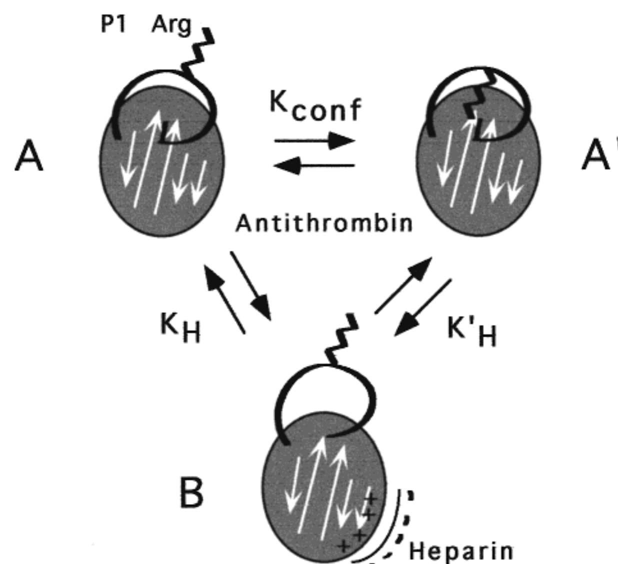
Despite the large reductions in the rate of association of the P1 antithrombin variants with thrombin and factor Xa, the stability of the complexes formed between the P1 Trp variant and these enzymes was similar to that of the wild-type inhibitor complexes as judged by the similar rate

constants for dissociation of the complexes. Such findings convincingly argue that P1 residue interactions with the proteinase S1 site are not involved in stabilizing these complexes and support the findings of previous studies which showed a repositioning of the P1 residue in the final covalent complex (*31*) and the lack of involvement of other reactive center loop residues in complex stabilization (*17*, *28*, *44*). These observations agree with the elucidated crystal structure of a serpin–proteinase complex which reveal trapping of the proteinase as a covalent acyl–enzyme complex following its translocation from an initial docking site to the opposite end of the serpin (*45*). The translocation is triggered by the proteinase cleaving the reactive center loop, allowing the loop and covalently linked proteinase to insert into β-sheet A of the serpin. The insertion disrupts reactive center loop interactions with the proteinase, expels the serpin P1 residue from the enzyme S1 site, and distorts the enzyme active-site region, thus accounting for the stability of the complex (*45*).

Our finding that the P1 tryptophan of the variant antithrombin underwent only minor changes in its fluorescence emission properties upon heparin activation indicates that the environment of the mutant P1 residue was minimally affected by activation. The observed P1 tryptophan fluorescence emission properties are indicative of a solvent-exposed residue in both native and activated states (*46*) and thus support similar solvent accessibilities of the P1 residue in the two states. Although a previous study observed that a P1 Arg → Cys antithrombin labeled with the environmentally sensitive fluorphore, NBD, underwent substantial increases in fluorescence upon heparin activation indicative of a change in environment (*5*), these fluorescence changes are shown in the accompanying paper to result from the relief of quenching of the fluorophore due to its interaction with the serpin body in the native inhibitor and not to involve any significant changes in the fluorophore lifetime (*47*). Such findings imply that the environment of the P1 residue is indeed similar in the native and heparin-activated serpin conformations and that the closer proximity of the P1 residue to the serpin body in the native state brought about through partial burial of the loop is likely responsible for a weak fluorophore interaction which minimally affects the environment or accessibility of the fluorophore.

Our studies have confirmed previous evidence in support of a P1 arginine interaction with the serpin body in native antithrombin by showing that a P1 Arg → His mutation enhances heparin binding affinity a modest but significant 2–3-fold (*11*, *13*), that loss of this affinity enhancement at lower pH correlates with the histidine becoming positively charged (*11*), and that other P1 residue mutations have similar small effects on heparin affinity (*32*). The enhanced heparin affinity of the P1 arginine variants is consistent with these mutations disrupting a P1 arginine intramolecular interaction which stabilizes the native low-heparin affinity conformation, thereby lowering the energy cost for activating the inhibitor to the high-heparin affinity conformation. However, the small extent to which heparin affinity was enhanced by the P1 arginine mutations implies that the stabilization of the native conformation by the P1 arginine interaction cannot be very large, i.e., that it should approximate 0.4–0.6 kcal/mol based on the 2–3-fold change. It follows that the energy difference between the two putative conformational states of the P1 arginine must also be small

Scheme 1: Revised Model for Heparin Modulation of Antithrombin P1 Residue Accessibility[a]



[a] The model depicts the P1 arginine of unactivated antithrombin (stick representation) in a mobile equilibrium between an exposed state (A, top left) and a state in which the P1 residue interacts with the serpin body (A′, top right). The interaction arises due to the partial burial of the reactive center loop into β-sheet A (depicted by the arrows) in the native conformation, bringing the P1 residue in close proximity to the serpin body. The weakness of the interaction ($K_{conf} \sim 2$) results in a significant fraction of the serpin existing in the exposed state and therefore being accessible to proteinases in the native state. Binding of heparin expels the reactive center loop from sheet A, fully exposing the P1 arginine side chain and increasing loop flexibility (B). The affinity of heparin for the antithrombin state in which the P1 arginine interacts with the serpin body is lower than its affinity for the state in which the P1 arginine is exposed ($K'_H > K_H$) due to the energy required to break the P1 arginine interaction.

and, therefore, interacting and noninteracting states of the P1 arginine are likely to exist in a mobile equilibrium in the unactivated inhibitor. This equilibrium in the favored loop-buried conformation of native antithrombin is distinct from the equilibrium between loop-buried and loop-expelled conformations which is the basis for allosteric activation of the serpin by heparin (*12*, *48*). Expulsion of the reactive center loop from β-sheet A upon heparin activation could make the P1 arginine interaction with the serpin body less energetically favorable and shift the P1 arginine equilibrium toward the noninteracting state which is reactive with proteinases. The low-energy stabilization of the P1 arginine intramolecular interaction can account for the small effects of conformational activation of the loop by heparin pentasaccharide on the recognition of proteinases other than factor Xa which appear to interact only with the loop, i.e., trypsin and thrombin. However, the observation of somewhat larger effects of conformational activation of the loop on thrombin recognition in the case of antithrombin variants engineered to have a more favorable thrombin recognition sequence (*17*) may be ascribed to the greater flexibility of the loop after its expulsion from sheet A and consequent ability of a more extended sequence to better fit the enzyme active site (*17*).

In conclusion, our findings suggest a revised model for the role of the P1 arginine residue of antithrombin in determining the serpin's specificity for its target enzymes (Scheme 1). In this model, the P1 arginine exists in accessible

(A) and inaccessible (A′) states in the native serpin conformation due to a weak ionic or hydrogen bonding interaction between the P1 arginine and the serpin body brought about by their proximity in this conformation. The two states are nearly equally populated and in a mobile equilibrium due to the low energy barrier for breaking the P1 arginine interaction. As a result, the P1 residue is largely accessible in the native conformation. Activation of the serpin by heparin increases the level of exposure of the reactive center loop and its flexibility, thereby favoring the fully accessible state of the P1 arginine side chain (B). Reactivity with proteinases is only marginally enhanced by the activation because of the substantial fraction of the serpin already with an accessible P1 residue. Part of the enhancement may additionally arise from the increase in loop flexibility. According to this model, the P1 residue plays the expected critical role in recognizing target enzymes, but this role is similar for both native and activated states of the serpin and thus does not contribute significantly to the enhanced reactivity of the serpin with proteinases when it is activated by heparin. It follows that determinants other than the P1 residue are principally responsible for enhancing the specificity of antithrombin for its target proteinases when the serpin is activated by heparin.

ACKNOWLEDGMENT

We thank Peter Gettins and Ingemar Björk for their helpful comments on the manuscript.

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BI002933D