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Mutation of the H-helix in Antithrombin Decreases Heparin Stimulation of Protease Inhibition

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Abstract

Blood clotting proceeds through the sequential proteolytic activation of a series of serine proteases, culminating in thrombin cleaving fibrinogen into fibrin. The serine protease inhibitors (serpins) antithrombin (AT) and protein C inhibitor (PCI) both inhibit thrombin in a heparin-accelerated reaction. Heparin binds to the positively charged D-helix of AT and H-helix of PCI. The H-helix of AT is negatively charged, and it was mutated to contain neutral or positively charged residues to see if they contributed to heparin stimulation or protease specificity in AT. To assess the impact of the H-helix mutations on heparin stimulation in the absence of the known heparin binding site, negative charges were also introduced in the D-helix of AT. AT with both positively charged H- and D-helices showed decreases in heparin stimulation of thrombin and factor Xa inhibition by 10 and five-fold respectively, a decrease in affinity for heparin-sepharose, and a shift in the heparin template curve. In the absence of a positively charged D-helix, changing the H-helix from neutral to positively charged increased heparin stimulation of thrombin inhibition 21-fold, increased heparin affinity and restored a normal maximal heparin concentration for inhibition. (Word Count = 187)

Keywords

thrombin; factor Xa; thrombomodulin; coagulation; serpin

1. Introduction

The blood coagulation cascade consists of the sequential proteolytic activation of serine proteases. The cascade is regulated in part by heparin binding serine protease inhibitors (serpins) including antithrombin (AT) and protein C inhibitor (PCI) [1,2]. Serpins function by the insertion of a reactive site loop into the active site of the target serine protease. When the protease cleaves the loop, the serpin undergoes a conformational change trapping the protease in an inactive complex [3]. Antithrombin, PCI, heparin cofactor II (HCII), protease nexin I (PN1) and plasminogen activator inhibitor 1 (PAI1) are all members of a sub-class of serpins that bind to the sulfated glycosaminoglycan heparin through positively charged alpha helices,

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in most cases the D-helix [4]. The heparin binding domain of AT resides on or near the D-helix (Figure 1): K11, R13, R24, R47, K114, K125 and R129, as determined by site directed mutagenesis studies [5–9]. Previous experiments with AT mutants K125Q, K125M and R129M showed decreases in heparin binding by AT, but no significant reduction in AT inhibition of thrombin in the presence of heparin, or factor Xa in the presence of H5 [7,10–12]. However, the simultaneous mutation of K125 and R129 to alanine or glutamate blocks H5 stimulation of thrombin and factor Xa inhibition [13]. The role of these residues in heparin binding has been confirmed by co-crystallization of trimolecular complexes of thrombin, heparin and AT [14,15]. Among the heparin-binding serpins, PCI is unique in that heparin binds to the positively charged H-helix residues (Figure 1) R269, K270, K276 and K277 [16]. Alignment of the D and H-helices of the five heparin-binding serpins reveals that most have a positively charged D-helix with the exception of PCI which has a positively charged H-helix. The H-helices of PAI1, PN1 and HCII are all net positive, while the H-helix of AT contains only negatively charged residues which are in the same position as the heparin-binding residues in the H-helix of PCI (Figure 2).

The mechanism by which heparin stimulates serpin activation also differs between AT and PCI. When there is no cofactor present, PCI and AT inhibit thrombin with second-order rate constants (k_2) of $18 \times 103\ M^{-1}s^{-1}$ and $9 \times 10^3\ M^{-1}s^{-1}$ respectively [1,4,17]. Heparin binds to exosite II of thrombin and the D- or H-helix of the serpin, forming a bridge and increasing inhibition by 30-fold with PCI and 2000-fold with AT [18,19]. Heparin binding to the D-helix also causes a conformational change in the reactive site loop of AT that culminates with the exposure of both the P1 Arg of AT and an exosite on the serpin for interaction with coagulation proteases [5,6,20–25]. This conformational change contributes to stimulation of thrombin and factor Xa inhibition by 2 and 200-fold respectively [19,24,25]. In contrast, the reactive site loop of PCI is very flexible and always exposed to proteolytic attack, there is also no apparent heparin induced conformational change in PCI [26].

In addition to protease inhibition by serpins, coagulation is regulated by feedback inhibition of thrombin production. Some of the thrombin generated during coagulation binds to the endothelial cell receptor thrombomodulin (TM). The TM-bound thrombin recognizes protein C as a substrate, proteolytically generating activated protein C (APC). APC in turn proteolytically degrades factors Va and VIIIa, cofactors used in thrombin production [27]. In this manner, thrombin binding to TM activates an anticoagulant feedback loop by degrading cofactors needed for the further generation of thrombin. The physiological importance of this feedback loop has been demonstrated in TM deficient mice and patients with mutations in protein C or factor V [28–30]. The serpin specificity of thrombin is also changed when it binds to TM. AT inhibition of thrombin bound by TM is minimally changed except when chondroitin sulfate, a heparin-like polysaccharide, is present on TM [31,32]. When thrombin is bound to TM containing chondroitin sulfate, inhibition of the thrombin is 100-fold faster by PCI ($2.4 \times 10^6\ M^{-1}s^{-1}$) [17] than by AT ($2.8–3.6 \times 10^4\ M^{-1}s^{-1}$) [31,33]. Although PCI was initially identified as a specific inhibitor of APC, these results suggest that the main target for PCI in the vasculature may be the thrombin-TM complex. The EGF-like domains 5 and 6 of TM bind to the fibrinogen binding exosite-1 of thrombin orienting it such that a negatively charged region on EGF domain 4 of TM juxtaposes the positively charged H-helix of PCI [34]. If the H-helix of AT is mutated to resemble that of PCI, AT inhibition of thrombin-TM increases 70-fold, possibly due to interactions between negatively charged residues on EGF4 of TM and the now positively charged H-helix of AT [34].

While performing these experiments, we noted that AT with a positively charged H-helix also had a decrease in heparin stimulation of thrombin inhibition. To determine the role of a negatively charged H-helix on the function of AT, we mutated the D- and H-helices of AT to

the residues in the corresponding helices of PCI. These mutants were then used to determine the role of the AT H-helix in heparin dependent protease inhibition.

2. Materials and Methods

Plasmid Construction

Wild-type AT cDNA [35] was excised from the plasmid pKT218-AT (ATCC) with *Pst*I. A His-6 tag $(CAC)_6$ and *Bam*HI restriction site were added to the 3′ end of the cDNA by PCR amplification. The cDNA was ligated into pGEM3Zf(+) at the *Pst*I and *Bam*HI restriction sites. Plasmids were isolated from *E. coli* using a QIAprep Miniprep Kit (QIAgen, Valencia, CA).

Site-directed Mutagenesis

Specific mutations were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The following mutations were made; AT-D^{neg}, K125E, R129E; AT-H^{pos}, D309K, E310K, E312K, E313K; AT-H^{neut}, D309N, E310Q, E312Q, E313Q (Figure 2). All mutations were confirmed by DNA sequencing using a BigDye dideoxy sequencing kit. Double mutants were generated by sub-cloning the H-helix mutations into the baculovirus transfer vector pVL1392 (pVL-AT) containing the D-helix mutations using an *Nco*I site in the AT cDNA.

Expression and Purification of AT

pVL-AT containing either the wild-type or mutant AT cDNAs were cotransfected with Baculogold *Autographa californica* polyhedrosis virus DNA into *Sf9* (*Spodoptera frugiperda*) host insect cells in TMN-FH complete media using the Baculovirus Expression Vector System (PharMingen, San Diego, CA). Amplified viral stocks were used to infect shaking cultures of *Sf9 TriEx* cells (Novagen, Madison, WI) in Insect-Xpress serum-free medium (BioWhittaker) supplemented with 1mM L-glutamine, 2.5μg/ml fungizone and 5μg/ml gentamicin. Three days post-infection media containing recombinant AT was collected. Proteins with a His_6 tag were loaded onto a nickel agarose column equilibrated with buffer (0.3M NaCl, 50mM sodium phosphate, pH 7.4) containing 0.01M imidazole. The column was washed with buffer until baseline was established, and then washed with buffer containing 0.05M imidazole and the rAT eluted with buffer containing 0.25M imidazole. The fraction was adjusted to 10mM EDTA and diluted to 0.1M NaCl with 0.01M Tris. Proteins were then purified by heparin affinity chromatography. Unbound proteins were eluted with TBS (0.01M Tris, 0.02% azide, 0.1M NaCl, pH 7.4) and the rAT was then eluted with TBS containing 2M NaCl and dialyzed against TBS to remove excess salt. Fractions containing rAT were then loaded onto a Q-sepharose column and eluted in a 0-0.6M NaCl gradient. Fractions containing AT activity were dialyzed against TBS, concentrated and adjusted to 0.1% PEG before storing at −80°C.

Serine Protease Inhibition Assays

The rates of inhibition of thrombin in the presence and absence of heparin were measured using discontinuous assays under pseudo-first order rate conditions as described previously [17,36]. Proteins were diluted in TBS buffer containing 0.1 mg/ml BSA, 0.1% PEG 8000, and 0.01% azide to a final reaction volume of 50μl. Thrombin (0.5–2nM) and a 10-fold molar excess of AT were incubated together (15 sec. to 30 min. based on reaction rate), followed by the addition of 50 μl of Spectrozyme TH (SpTH) (American Diagnostica, Greenwich, CT) with 1 mg/ml Polybrene was added to the substrate to block further heparin binding during substrate development. For heparin template curve data, varying concentrations (0–1000 μg/ml) of unfractionated heparin (Sigma, St. Louis, MO) were used. Substrate was added and the microtiter plates were centrifuged for 10 min at 1000 × g to remove precipitated heparin/

polybrene complexes in the 100 and 1000 μg/ml heparin reactions and the supernatants transferred to new plates to measure absorbance. For subsequent assays in the presence of heparin the optimal concentration for each protein was used. Factor Xa assays were performed in a similar manner as the thrombin assays, using 2nM protease, 20nM AT or 100nM PCI, and 5nM heparin pentasaccharide (H5, provided by Steve Olson, University of Illinois-Chicago) or 5 μg/ml heparin for 5 to 60 minutes depending on reaction rate. Residual factor Xa activity was measured by addition of 50μl of 400μM Spectrozyme FXa. Second-order rate constants of thrombin inhibition were determined using the equation: $k_{obs} = (-\ln (A/A_0))/tI$, where A is the absorbance of the inhibited sample at 405nm, A_0 is the absorbance of uninhibited sample, t is time of reaction in seconds and I is the concentration of AT in M [17,36]. All assays were performed in triplicate in two to five separate experiments.

Stoichiometry of Inhibition Assays

Stoichiometry of inhibition (SI) was measured using 10nM thrombin incubated with AT alone or in the presence of heparin (5μg/ml). Assays without heparin were performed with 0.1 mg/ml polybrene in the buffer to neutralize any traces of heparin in the sample. AT concentrations ranged from 5 to 100nM. Assays with heparin were incubated for one hour and those with AT alone were incubated for 16 hours. Substrate was added as described above, residual thrombin activity plotted versus AT concentration and the x-intercept calculated by least-squares linear regression. Second order rate constants of thrombin inhibition were multiplied by their respective SI values to calculate the actual rate of inhibition in the presence or absence of heparin.

3. Results

Expression of AT

The wild type recombinant AT (rAT) and mutant proteins were successfully expressed in insect cells using the Baculovirus expression system. Immunoblots of the media from the co-transfections demonstrated that the rAT had not been degraded and the molecular weight of the rAT was 51 kD, less than the 58 kD for human plasma AT due to previously reported differences in glycosylation [37]. The rAT-H^{Pos} and rAT-H^{Neut} proteins were purified using heparin sepharose. The rAT-$D^{Neg}H^{Pos}$ and rAT-$D^{Neg}H^{Neut}$ proteins could not be completely purified with heparin sepharose due to a decrease in heparin binding, and a polyhistidine tag was added to the proteins, allowing for nickel agarose chromatography prior to heparin sepharose to improve purity. All rAT proteins eluted from heparin-Sepharose at greater than 1.0M NaCl with the following exceptions; rAT-H^{Pos} eluted at 0.6M NaCl, rAT-$D^{Neg}H^{Pos}$-His_6 eluted at 0.4M NaCl, and rAT-$D^{Neg}H^{Neut}$-His_6 eluted at 0.2M NaCl.

Inhibition of Thrombin

Plasma AT, rAT and the mutants displayed a traditional bell shaped heparin dependence of inhibition of thrombin with optimal thrombin inhibition at a heparin concentration of 5 μg/ml (Figure 3). The only exceptions were the rAT-H^{Pos} and rAT-$D^{Neg}H^{Neut}$-His_6 mutants, which had shifts in optimal heparin concentrations to 1 μg/ml and 10 μg/ml respectively. Wild-type rAT and plasma AT both showed approximately 3000-fold acceleration of thrombin inhibition in the presence of heparin (Table 1). Recombinant AT activity expressed was reduced 2.5-fold relative to purified human AT, consistent with previous results from baculovirus expression of AT [37,38]. rAT-His_6 showed a four-fold loss in heparin-stimulated thrombin inhibition when compared to rAT, mostly due to an increase in inhibition in the absence of heparin, and an increase in SI in the presence of heparin. This is consistent with the observation that addition of a C-terminal His tag on AT partially interferes with heparin stimulation of thrombin inhibition [39].

Generating an AT with positively charged D- and H-helices, rAT-H^{Pos}, led to a six-fold increase in inhibition of thrombin in the absence of heparin, and 10-fold decrease in thrombin inhibition in the presence of heparin relative to rAT. Changing the same H-helix residues to neutral amino acids led to Table 1 smaller two-fold changes in thrombin inhibition in the absence and presence of heparin (). Both mutants had slightly increased SI values relative to rAT, indicating that these mutants have increased reactivity with the protease in the substrate pathway of the reaction.

Changing D-helix residues to negatively charged residues in rAT-$D^{Neg}H^{Neut}$-His_6, did not affect inhibition in the absence of heparin, but abolished heparin binding and stimulation of thrombin inhibition, consistent with published results from the mutation K125E, R129E [13]. Adding a positively charged H-helix, rAT-$D^{Neg}H^{Pos}$-His_6, restored a 21-fold increase in thrombin inhibition with heparin. However, the rate is still 30-fold lower than that of rAT-His_6. Interestingly, the rate of thrombin inhibition by rAT-$D^{Neg}H^{Pos}$-His_6 was only two-fold less than that of rAT-H^{Pos}, which has an intact D-helix (Table 1)

Inhibition of Factor Xa

To ensure that the H-helix mutants were not affecting the conformation of the heparin binding domain of the D-helix, inhibition of factor Xa was measured in the presence of 0–5nM H5. The wild-type and H-helix mutant ATs all showed similar rates of inhibition of factor Xa with increasing concentration of H5 (Figure 4). In contrast the two D-helix mutants had no enhancement of inhibition of factor Xa in the presence of H5. This was also confirmed by measuring the stimulation of factor Xa inhibition of each AT mutant in the presence of H5. Proteins with a normal D-helix showed 30-fold stimulation while those with negatively charged D-helices had a 10-fold decrease in stimulation. These are not maximum stimulations of Xa inhibition, as only 5nM H5 was used. The rAT-H^{Pos} mutant also showed a two-fold increase in inhibition of factor Xa relative to rAT in the presence or absence of H5 (Table II).

Each AT mutant was also assayed for stimulation of factor Xa in the presence of full-length heparin. The trend was similar to that observed for heparin stimulation of thrombin inhibition (Table II). The wild-type ATs and rAT-H^{Neut} had similar fold stimulations in the presence of heparin, while rAT-H^{Pos} had a five-fold decrease in stimulation compared to the control. Changing D-helix residues to negatively charged residues in rAT-$D^{Neg}H^{Neut}$-His_6, did not affect factor Xa inhibition in the absence of heparin, but abolished heparin stimulation of factor Xa inhibition. Adding back a positively charged H-helix, rAT-$D^{Neg}H^{Pos}$-His_6, restored a six-fold increase in factor Xa inhibition with heparin.

4. Discussion

PCI is unique among serpins in binding heparin through the H-helix, while the other heparin-binding serpins bind heparin through the D-helix. AT is unique in that its H-helix residues are negatively charged, while the other heparin-binding serpins have net positively charged H-helixes. In addition to these structural differences, AT is primarily an anticoagulant, inhibiting thrombin, factor IXa and factor Xa, while PCI is primarily a procoagulant in the blood, inhibiting both the formation and activity of APC and thrombin-TM. Our results suggest that the location of the heparin-binding sites may play an important role in determining the target protease specificity and thus the procoagulant and anticoagulant functions of these two serpins in the clotting cascade.

In the absence of heparin, PCI is better than AT at inhibiting thrombin and factor Xa. This is thought in part to be due to PCI having a more extended reactive site loop, and the orientation of the P1 Arg away from the body of the serpin (Figure 1). However, heparin stimulates thrombin inhibition by AT >2000-fold, compared with a 30-fold increase for PCI. The trend

was even more striking with factor Xa, where heparin stimulated AT inhibition 700-fold, yet actually decreased PCI inhibition three-fold. When heparin binds to the positively charged D helix of AT the helix elongates, extending the reactive site loop and exposing the P1 Arg which stimulates thrombin and Xa inhibition. Olson and Chuang have speculated that the reactive loop sequence of AT is not optimal for recognition by thrombin and factor Xa as a mechanism to prevent recognition by APC, thus ensuring that AT functions as an effective anticoagulant [25]. Thus, when heparin binds to the D-helix of AT, the resulting heparin-induced conformational change and the bridging effect of long chain heparins are responsible for switching AT from a weak to a strong anticoagulant. In contrast, when heparin binds to the H-helix of PCI, no major conformational change is thought to occur, and the only stimulation of thrombin inhibition is through a bridging mechanism, favoring PCI's role as a procoagulant. The recent crystal structure of intact PCI (Figure 1) reveals an exposed loop in the absence of heparin, supporting this theory (26).

When the H-helix of AT was changed to more closely resemble the H-helix of PCI, in AT-H^{pos}, heparin acceleration of thrombin inhibition was reduced 10-fold, and factor Xa inhibition five-fold, to values closer to that of PCI. Making these same H-helix residues neutral, AT-H^{neut}, decreased heparin stimulation of thrombin two-fold and had no effect on factor Xa inhibition, suggesting that the change was mostly due to the addition of positive charges to the H-helix of AT, rather than the loss of negative charges. It is plausible that a new competitive heparin-binding site was created on the H-helix of AT, because the heparin template curve for the AT-H^{pos} mutant shifted slightly to the left, indicating that less heparin was needed to achieve maximal heparin stimulation. This was also consistent with a decrease in maximal rate of thrombin inhibition, as heparin bound to the H-helix may not be able to bind to the D-helix, thus blocking extension of the reactive site loop of AT.

To block heparin stimulation of AT through binding to the D-helix, mutation were generated in a rAT-His_6 background. The introduction of a negatively charged N-terminal tag in AT-His_6 improved the reactivity of the serpin with thrombin in the absence of heparin, possibly through its interaction with a complementary site of the protease. However, such an interaction does not appear to occur for the mutant AT with factor Xa, possibly accounting for the differential extent of the cofactor effect of heparin in stimulating the AT-His_6 inhibition of these proteases. As expected, the mutations K125E and R129E, in rAT-$D^{Neg}H^{Neut}$-His_6, resulted in decreased heparin stimulation of thrombin inhibition, no H5 stimulation of factor Xa inhibition, and a shift in the heparin concentration for optimal thrombin inhibition, indicating that more heparin was necessary to stimulate thrombin inhibition. Adding a positively charged H-helix to this D-helix mutant (rAT-$D^{Neg}H^{Pos}$-His_6) restored normal optimal heparin concentrations for thrombin inhibition. Heparin stimulation of thrombin inhibition also increased to 20-fold, similar to that seen for PCI, which also has a negatively charged D-helix and positively charged H-helix. These rates of inhibition are consistent with a new heparin binding site on the H-helix, that can increase thrombin inhibition through a bridging mechanism, as seen with PCI, but not through the traditional conformational change seen in AT.

An alternative explanation is that a negatively charged H-helix in AT reduces the rate of inhibition of thrombin alone in the absence of heparin by a charge-charge repulsion mechanism between negatively charged residues in the AT H-helix and possibly the 60-D loop of thrombin. In support of this hypothesis, the structure of the ternary AT-thrombin-heparin complex shows that Asp-60 of thrombin is very close to Glu-313 of AT (~7Å). Thus, a repulsive interaction between these two negatively charged residues may also account for the slower reactivity of the serpin with thrombin in the absence of heparin.

AT is unique among the heparin binding serpins in that it has a negatively charged H-helix. Reversing the charge of this helix to resemble that of PCI decreases heparin stimulation of thrombin inhibition and increases the rate of inhibition of thrombin-TM. Both of these changes cause AT-H^{pos} to behave more like PCI than AT. Thus the negative charges on the H-helix of AT may add to its anticoagulant activity by preventing heparin binding to the H-helix, and by repelling the EGF4 domain of TM and thus not inhibiting APC formation.

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Abbreviations

AT
: antithrombin

PCI
: protein C inhibitor

TM
: thrombomodulin

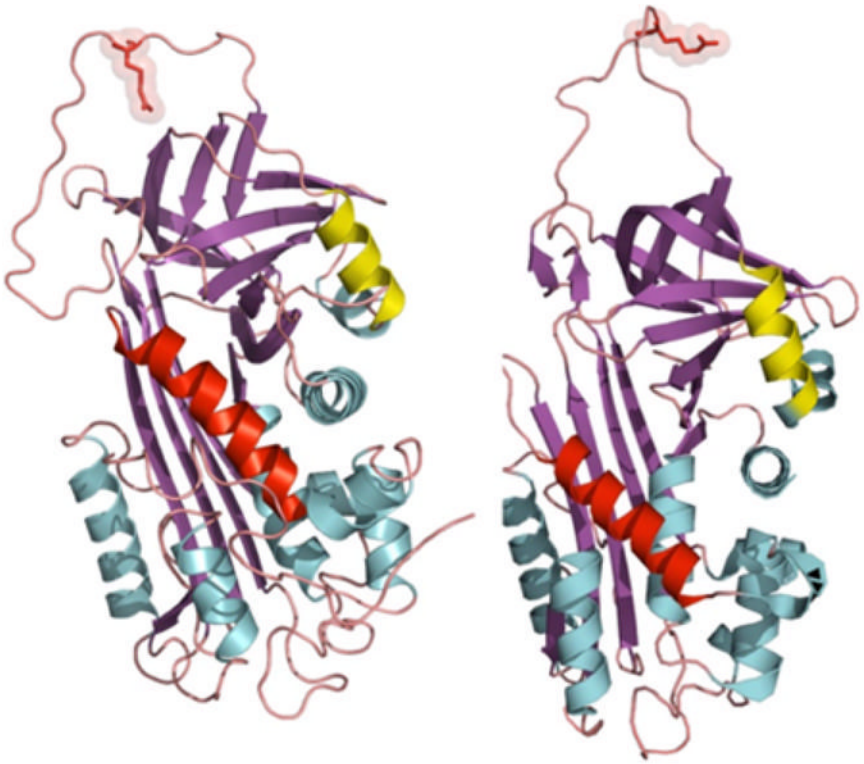

Figure 1.
Crystal structures of intact AT, PDB entry 1AZX (left) and PCI, PDB entry 2OL2 (right) with D-helices highlighted in red and H-helices in yellow. The P1 Arg on the reactive site loop is shown in stick with van der Waal radii.

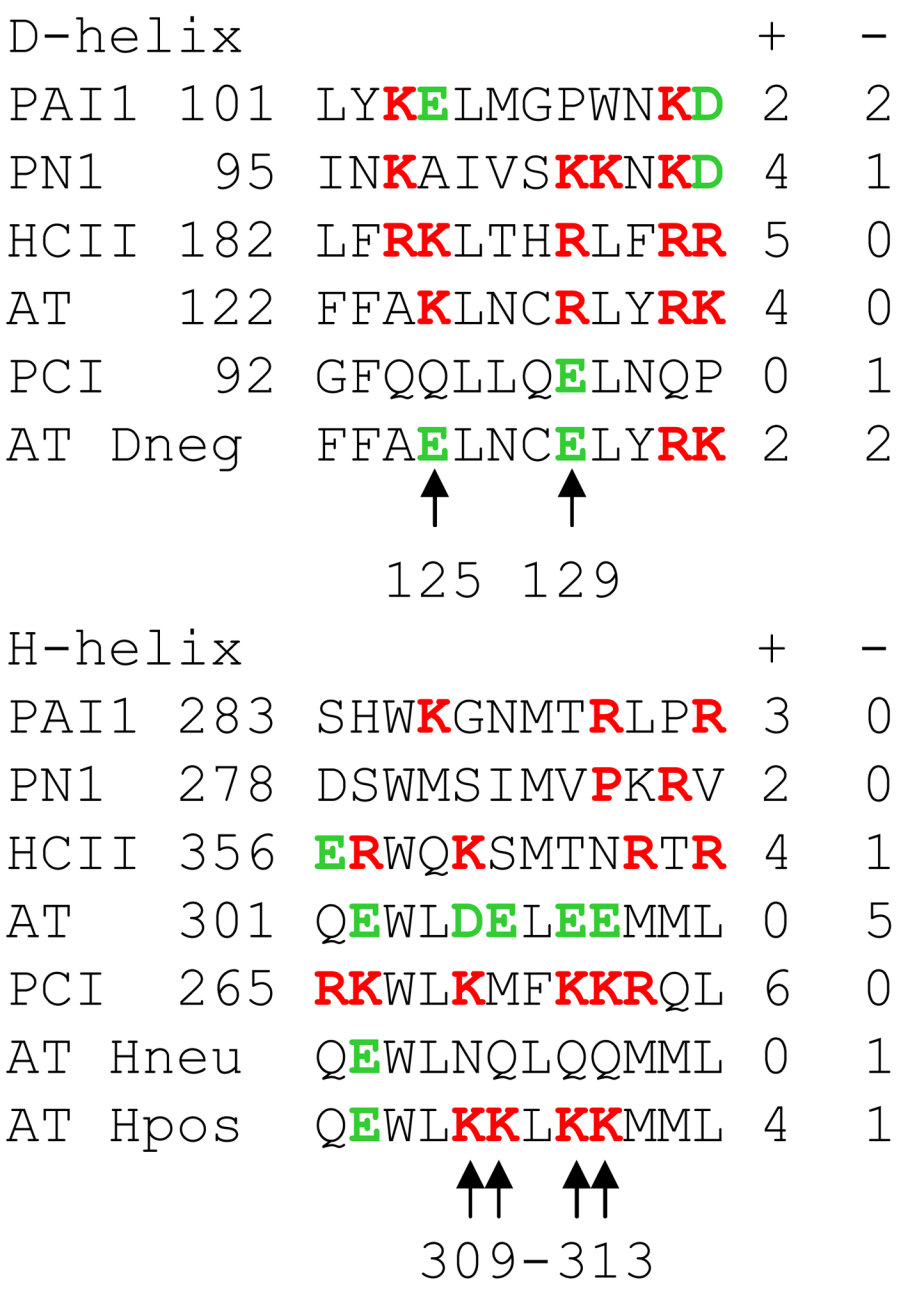

Figure 2.
Alignments of D- and H-helices of the heparin-binding serpins, PAI1, PN1, HCII, AT, and PCI. Conserved residues are blue, positively charged are red and negatively charged are green. The AT residues changed in this study are indicated by arrows. The columns to the right of each alignment indicate the total numbers of positive and negative residues in each aligned sequence.

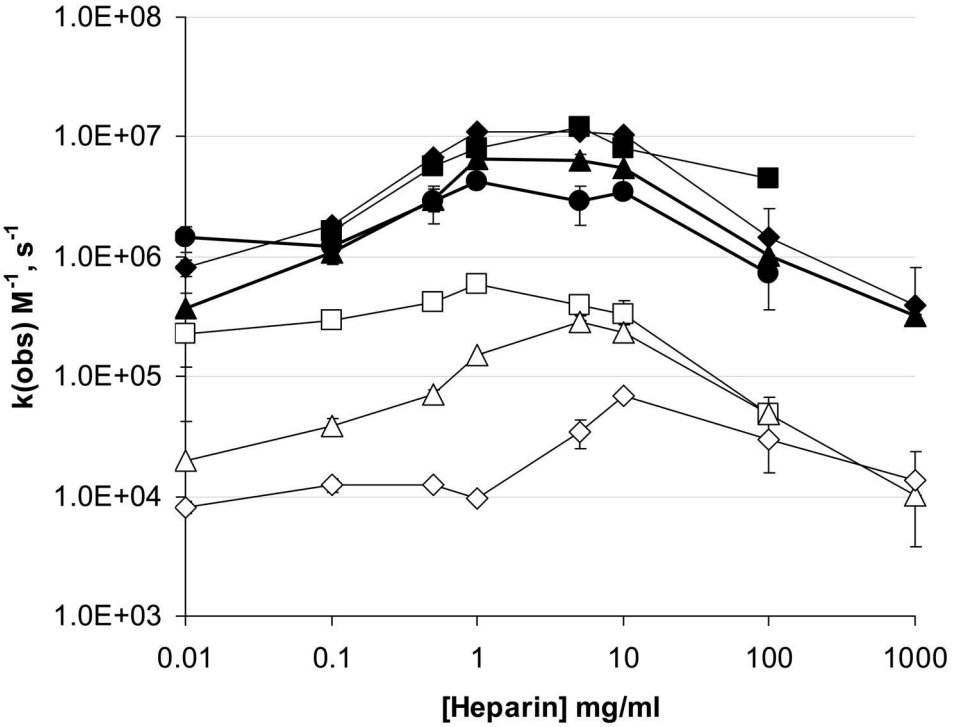

Figure 3.

Thrombin (1nM) was incubated with 10nM of each of the following antithrombins in the presence of increasing concentrations of heparin; human AT( ), wild type rAT (■), wild type rAT-His_6 (▲), rAT-H^{neut} (●), rAT-H^{pos} (□), rAT-$D^{neg}H^{pos}$-His_6 (△), rAT-$D^{pos}H^{neut}$-His_6 (◇). Reactions were stopped after 15 seconds to 15 minutes at room temperature by the addition of Spectrozyme TH and polybrene, final concentrations 0.2 mM and 0.1 mg/ml respectively. Pseudo first-order rate constants (k_{obs}) were calculated from the remaining activity of enzymes as described under “Experimental Procedures”

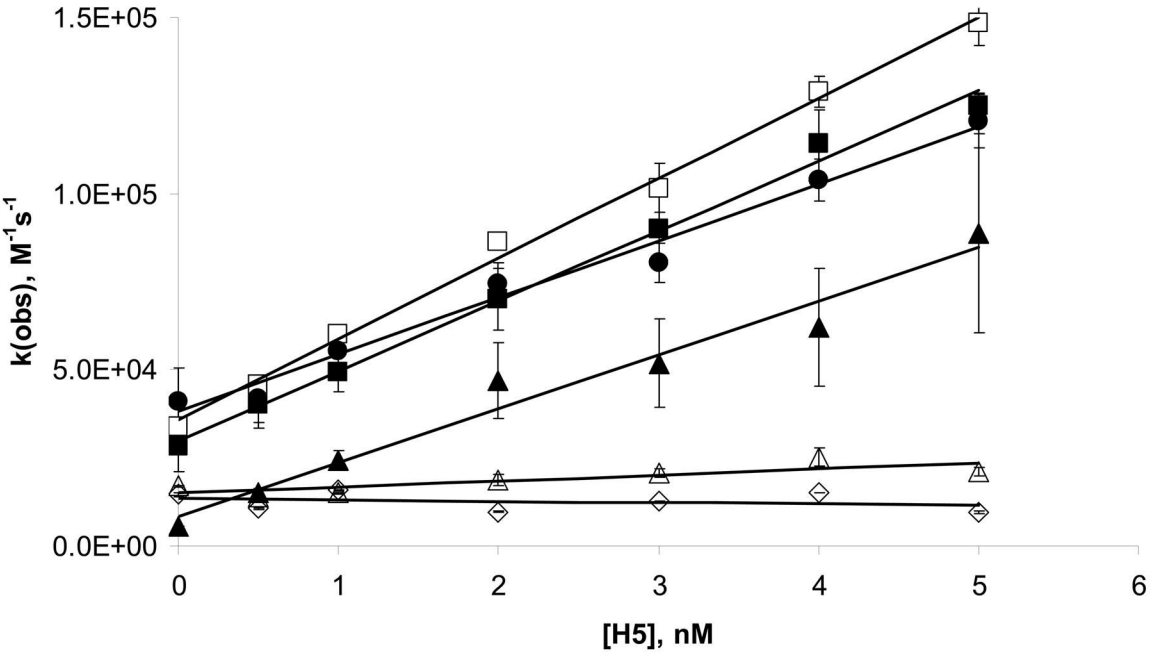

Figure 4.

Factor Xa (2 nM) was incubated with 20nM of each of the following antithrombins in the presence of increasing concentrations of heparin pentasaccharide (H5); wild type rAT (■), wild type rAT-His_6 (▲), rAT-H^{neut} (●), rAT-H^{pos} (□), rAT-$D^{neg}H^{pos}$-His_6 (△), rAT-$D^{pos}H^{neut}$-His_6 (◇). Reactions were stopped after 5 minutes at room temperature by the addition of Spectrozyme FX and polybrene, final concentrations 0.2 mM and 0.1 mg/ml respectively. Pseudo first-order rate constants (k_{obs}) were calculated from the remaining activity of enzymes as described under “Experimental Procedures”.

Table I

Second-order inhibition rate constants (in $M^{-1}sec^{-1}$) for the reaction of thrombin with wild type and mutant antithrombins in the presence and absence of heparin

	No cofactor			Heparin			
	k_2[a]	SI[b]	$k_2 \times SI$	k_2	SI	$k_2 \times SI$	+hep/−hep
PCI	1.8×10^4			2.7×10^5			15
hAT	9.3 ± 0.3[c] $\times 10^3$	1.1	1.0×10^4	$2.5 \pm 0.4 \times 10^7$	1.2	3.0×10^7	2885
rAT	$3.6 \pm 1.3 \times 10^3$	1.1	4.0×10^3	$1.1 \pm 0.3 \times 10^7$	1.1	1.2×10^7	3060
rAT-H^{pos}	$2.0 \pm 0.5 \times 10^4$	1.3	2.6×10^4	$8.2 \pm 0.6 \times 10^5$	1.7	1.4×10^6	54
rAT-H^{neut}	$7.4 \pm 2.9 \times 10^3$	1.4	1.0×10^4	$4.5 \pm 0.2 \times 10^6$	1.5	6.7×10^6	649
rAT His_6	$2.6 \pm 0.8 \times 10^4$	1.2	3.1×10^4	$1.4 \pm 0.5 \times 10^7$	1.6	2.3×10^7	718
rAT-D^{neg} H^{pos}-His_6	$2.0 \pm 0.9 \times 10^4$	1.5	2.9×10^4	$3.3 \pm 1.0 \times 10^5$	1.9	6.3×10^5	21
rAT-$D^{neg}H^{neut}$-His_6	$2.1 \pm 0.8 \times 10^4$	1.0	2.1×10^4	$2.2 \pm 0.5 \times 10^4$	1.3	2.8×10^4	1

[a] All thrombin inhibition assays are described under “Experimental Procedures”

[b] Stoichiometry of Inhibition

[c] All values are the averages of at least three assays each done in triplicate ± S.D. values. For clarity S.D. values were not multiplied by SI.

Table II

Second-order inhibition rate constants (in $M^{-1}sec^{-1}$) for the reaction of factor Xa with wild type and mutant antithrombins in the presence and absence of heparin pentasaccharide or heparin

	No cofactor k_2[a]	H5 k_2	Heparin k_2	+ H5/no cofactor	+hep/no cofactor
PCI	5.4±0.2[b] × 10^3	5.1±0.2 × 10^3	2.6±0.2 × 10^3	0.9	0.5
rAT	2.2±1.0 × 10^3	5.1±0.0 × 10^4	1.5±0.1 × 10^6	22.6	682.7
rAT-H^{pos}	4.2±1.9 × 10^3	1.2±0.3 × 10^5	5.7±2.9 × 10^5	28.2	136.6
rAT-H^{neut}	1.1±0.1 × 10^3	6.8±1.1 × 10^4	6.6±1.6 × 10^5	79.6	611.4
rAT His_6	1.9±0.5 × 10^3	5.9±1.8 × 10^4	1.1±0.1 × 10^6	31.4	560.1
rAT-D^{neg} H^{pos}-His_6	1.3±0.2 × 10^3	4.9±0.2 × 10^3	1.4±0.0 × 10^4	3.0	9.3
rAT-$D^{neg}H^{neut}$-His_6	1.9±0.4 × 10^3	2.5±1.7 × 10^3	2.2±1.6 × 10^3	1.6	1.4

[a] All factor Xa inhibition assays are described under “Experimental Procedures”.

[b] All values are the averages of at least three assays each done in triplicate ± S.D. values.