Antithrombin mainly inhibits factor Xa and thrombin. The reactive center loop (RCL) is crucial for its interactions with its protease targets and is fully inserted into the A-sheet after its cleavage, causing translocation of the covalently linked protease to the opposite end of the A-sheet. Antithrombin variants with altered RCL hinge residues behave as substrates rather than inhibitors, resulting in stoichiometries of inhibition greater than one. Other antithrombin residues have been suggested to interfere with RCL insertion or the stability of the antithrombin–protease complex, but available crystal structures or mutagenesis studies have failed to identify such residues. Here, we characterized two mutations, S365L and I207T, present in individuals with type II antithrombin deficiency and identified a new antithrombin functional domain. S365L did not form stable complexes with thrombin or factor Xa, and the I207T/I207A variants inhibited both proteases with elevated stoichiometries of inhibition. Close proximity of Ile-207 and Ser-365 to the inserted RCL suggested that the preferred reaction of these mutants as protease substrates reflects an effect on the rate of the RCL insertion and protease translocation. However, both residues lie within the final docking site for the protease in the antithrombin–protease complex, supporting the idea that the enhanced substrate reactions may result from an increased dissociation of the final complexes. Our findings demonstrate that the distal end of the antithrombin A-sheet is crucial for the last steps of protease inhibition either by affecting the rate of RCL insertion or through critical interactions with proteases at the end of the A-sheet.

Serine protease inhibitors (serpins) are a superfamily of proteins that control proteases involved in inflammation, coagulation, and fibrinolytic pathways (1). Inhibitory serpins exert control over their target proteases by an unusual branched pathway suicide substrate mechanism. Antithrombin inhibits its target proteases by this branched pathway in a manner that preferentially promotes the formation of a stable inhibitory covalent complex with the target protease instead of allowing the proteolytic cleavage of the inhibitor (2). This serpin requires activation by heparin to achieve a physiologically significant rate of inhibition of its target proteases. Heparin activates antithrombin by two mechanisms. In one mechanism, heparin serves as a polysaccharide bridge to promote the encounter between protease and antithrombin, whereas in the second mechanism, the serpin is activated through conformational changes, which enhance reactivity with protease and involve several exosite interactions (3–6). The first mechanism is dominant with the protease, thrombin, and the second selectively enhances antithrombin reactivity with factor Xa and factor IXa. In the serpin inhibitory mechanism, target proteases initially recognize and bind a substrate amino acid sequence in the reactive center loop (RCL) of the serpin, the P1 (Arg-393) residue being the most critical determinant of this sequence (2, 7–9). The subsequent cleavage of the RCL by the protease to form the acyl-intermediate is followed by a major RCL conformational change, in which the N-terminal fragment of the cleaved RCL is inserted into the center of the A-sheet with the protease covalently attached. This translocates the protease to the opposite end of the serpin and inactivates the enzyme through distortion of the active site. The high efficiency of the RCL conformational change ensures that the stoichiometry of inhibition (SI) is always close to one. However, this mechanism makes serpins highly sensitive to mutations, predominantly those affecting the RCL: Gly-392 (P2), Ala-384 (P10), Ser-382 (P12), Glu-381 (P13), or Ser-380 (P14) antithrombin residues (10–14). Such mutations can thus reduce the rate of loop insertion and result in the inefficient inhibition of the protease, as reflected by increased SI values (2). Other residues may be involved in this mechanism of inhibition, and their modification could similarly increase the stoichiometry of inhibition.
The routine analysis of patients with blood clotting disorders that have been found to be associated with antithrombin deficiencies has identified many natural antithrombin variants. Those antithrombin deficiencies are divided into type I and type II. Type II antithrombin deficiencies are provoked by variants secreted to the plasma with an impaired anticoagulant activity. Type II variants have helped identify functional domains of this key anticoagulant, such as the RCL, the heparin-binding site (HBS), or the C-sheet, in which mutations can cause a variety of functional defects (15–19). We aimed to identify new functional domains of antithrombin by evaluating cases with type II deficiency with mutations outside these classical functional domains (RCL, HBS, or C-sheet). We report the first finding of two natural mutations at the end of the A-sheet distal from the RCL in the antithrombin structure that cause significant impairment of antithrombin function, mainly increasing the stoichiometries of inhibition. Essentially, such mutations point out a new serpin functional domain in which natural mutations might have clinical relevance not only for antithrombin, but for other inhibitory serpins as well.

Results
Identification of patients with type II antithrombin deficiency: Clinical, genetic, and analytical features

Among all the mutations identified in the 142 patients with antithrombin deficiency characterized by our laboratory, 51 were classified as type II antithrombin deficiencies, defined by the presence of variants in plasma with reduced anticoagulant activity. Five of them were not clearly located in key functional domains or regions of antithrombin: I207T; L340F; S349P; S365L; and H369Y. We selected two cases, S365L and I207T, as these residues are close in the tertiary structure of antithrombin, despite being distant in the primary sequence. Specifically, these residues are located at the end of sheet A most distal from the RCL in the antithrombin structure that cause significant impairment of antithrombin function, mainly increasing the stoichiometries of inhibition. Essentially, such mutations point out a new serpin functional domain in which natural mutations might have clinical relevance not only for antithrombin, but for other inhibitory serpins as well.

Formation of complexes by S365L mutant

Recombinant expression of S365L in HEK-EBNA cells confirmed the type II deficiency, because the variant was secreted (20). Contrasting the type I deficiency profile, our patient showed heparin cofactor anti-Xa and anti-IIa activity values severely reduced by 54 and 50%, respectively, but only slightly reduced antigen levels (71%). Moreover, a relatively high proportion of disulfide-linked antithrombin dimers was detected in plasma by SDS-PAGE under nonreducing conditions and Western blotting of recombinant antithrombins from HEK-EBNA cells transfected with wild-type (C) and S365L mutant antithrombin plasmids (S365L). Native AT and disulfide-linked dimers are shown by arrows. Notably, the S365L variant showed a higher proportion of disulfide-linked dimers compared to the wild-type variant. It is important to note that these dimers are not formed by the wild-type protein under the same conditions.
to the conditioned medium (Fig. 2B). Moreover, in agreement with the data from the patient, disulfide-linked dimers were observed in the conditioned medium of HEK-EBNA cells transfected with this mutant (Fig. 2B). These data suggest a conformational instability of this mutant antithrombin. In fact, the protein precipitated during the spin filter concentration after the purification process. Therefore, we evaluated its function by analyzing the formation of complexes of secreted antithrombin with target proteases. This variant did not form complexes with FXa or FIIa and behaved as a substrate with both proteases in the absence or presence of heparin, as judged by the identification of cleaved antithrombin in SDS-PAGE under nonreducing conditions (Fig. 3, A and B).

Effects of the I207T and I207A mutations on the reactivity of antithrombin

The wild-type control antithrombin (corresponding to β-antithrombin) and I207T and I207A variants were expressed and secreted in an insect cell expression system. I207A was produced to evaluate the effect of the mutation to a different amino acid. After their purification, kinetic analysis confirmed that the I207T mutant showed a 2–3-fold reduced apparent second order rate constant for inhibition (k_{app}) of thrombin and factor Xa relative to the control antithrombin in both the absence and the presence of heparin. Stoichiometric end point titrations revealed that the reduced apparent inhibition rates resulted largely from an elevated SI that was greater in the presence than in the absence of heparin. This was indicated from the partial or complete normalization of the inhibition rates after multiplying k_{app} by the SI to correct for the increased reaction along the substrate pathway (Table 1). Such normalization implies that the effect of the mutation is to increase the partitioning of the acyl-intermediate along the substrate pathway without affecting the rate at which the acyl-intermediate is trapped along the inhibitory pathway (21).

Contrasting the mild effect of the I207T mutation on antithrombin reactivity, the mutation of Ile-207 to alanine resulted in apparent rate constants of inhibition of both proteases (k_{app}) that were severely decreased in both basal and activated states (Table 1). This again appeared to largely reflect elevated inhibition stoichiometry values with both proteases, because the SIs were drastically increased in parallel with the decreases in k_{app}. Indeed, correction of k_{app} for the elevated SIs resulted in second-order rate constants for inhibition of FIIa that were close to those of the control antithrombin, consistent with the decrease in k_{app} arising from an enhanced partitioning of the acyl-intermediate along the substrate pathway. However, this correction yielded second-order inhibition rate constants with factor Xa that exceeded the rate constants of control antithrombin by 5–8-fold in the absence or presence of heparin (Table 1). The higher corrected rates for the latter mutant inhibitor reaction suggested the possibility that the measured SI overestimated the true SI. This could occur if the mutation affected the stabil-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Association rate constants (k_{app} × SI) and inhibition stoichiometries (SI)</th>
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<tbody>
<tr>
<td></td>
<td>k_{app} × SI</td>
</tr>
<tr>
<td></td>
<td>FIIa</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>(1.1 ± 0.2) × 10^{4}</td>
</tr>
<tr>
<td>+ UFH</td>
<td>(1.6 ± 0.1) × 10^{5}</td>
</tr>
<tr>
<td>I207T</td>
<td>(3.8 ± 0.2) × 10^{3}</td>
</tr>
<tr>
<td>+ UFH</td>
<td>(4.9 ± 0.3) × 10^{4}</td>
</tr>
<tr>
<td>I207A</td>
<td>(2.9 ± 0.9) × 10^{3}</td>
</tr>
<tr>
<td>+ UFH</td>
<td>(6.1 ± 0.8) × 10^{4}</td>
</tr>
</tbody>
</table>

Control antithrombin (β-antithrombin, S137A) and I207T and I207A variant reactions with FIIa and FXa. Values were measured as described under “Experimental procedures.” —, absence of cofactor; H5, pentasaccharide; UFH, unfractionated heparin; k_{app}, apparent second order rate constant for inhibition; SI, stoichiometry.


**New functional domain in antithrombin**

Table 2

<table>
<thead>
<tr>
<th>Recombinant AT</th>
<th>$K_D$ (mM)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>62.4 ± 1.0</td>
<td>32.0</td>
</tr>
<tr>
<td>I207T</td>
<td>39.1 ± 1.4</td>
<td>40.0</td>
</tr>
<tr>
<td>I207A</td>
<td>30.6 ± 1.0</td>
<td>38.0</td>
</tr>
</tbody>
</table>

Heparin affinity of I207T and I207A variants

Intrinsic fluorescence analysis revealed that purified I207T and I207A mutants exhibited a slightly increased heparin pentasaccharide affinity in comparison with wild-type antithrombin. The dissociation constant of these variants was lower than control antithrombin (Table 2). This suggested that the mutations caused a slight activation of the inhibitor in the absence of heparin, a finding observed with other antithrombin variants (6).

Thermodynamic stability of I207T and I207A mutants

The thermal denaturation assay demonstrated that mutations affecting Ile-207 provoked structural instability. The $T_m$ of native control antithrombin was 57.5 ± 0.1 °C, whereas both mutants presented lower denaturation temperatures (I207T: 54.7 ± 0.1 °C and I207A: 56.0 ± 0.2 °C).

Discussion

Serpins share a common molecular architecture and mechanism of protease inhibition. As a serpin, antithrombin inhibits its target blood coagulation proteases by the standard branched pathway suicide substrate mechanism of inhibition. However, this serpin requires activation by the cofactor, heparin, to enable it to specifically recognize its protease targets and achieve a physiologically significant rate of inhibition. Heparin activation of antithrombin provides new exosites on the serpin and a bridging site on the heparin cofactor to augment the initial docking of protease with the serpin RCL and promote acylation of the RCL P1-P1' bond. The protease is inhibited as with other serpin reactions as a result of acylation triggering a rapid RCL conformational change that traps the acyl-intermediate by deforming the RCL-linked protease at the distal end of sheet A. However, a fraction of the acyl-intermediate may escape this trapping by decaying before the conformational distortion of the protease is complete, resulting in the release of RCL-cleaved antithrombin as a substrate.

The identification and analysis of natural mutations in patients with antithrombin deficiency have assisted the description of key functional domains or residues of this anticoagulant (15–18, 22). Thus, mutations at the RCL, HBS, and the C-sheet are responsible for the three subtypes of antithrombin type II deficiency. Type II mutations usually do not affect the folding and secretion of the antithrombin variant, but impair the protease reactivity, heparin activation, or both. Multiple data, from X-ray crystallographic and biochemical studies of antithrombin variants mutated in P1 (Arg-393) or flanking residues (Gly-392, Ala-384, S382, Gln-381, and Ser-380) have demonstrated that RCL residues are crucial not only for the initial docking and acylation steps, but also for the partitioning of the acyl-intermediate complex in favor of the stable covalent complex. The latter defects result from the mutations interfering with the RCL conformational change that is responsible for trapping of the acyl-intermediate, i.e., they delay RCL insertion into the A-sheet and the concomitant translocation of the RCL-linked protease to the opposite end of this sheet. These mutations mostly located at the RCL hinge region cause variable proportion of the serpin to react as a substrate for the target protease (10–14, 23).

In this study, the analysis of natural mutations that render variants with impaired function (type II) has identified a new functional region in serpins relevant for completing the inhibitory process. These mutations map to a region of the serpin at the distal end of the A-sheet that represents the final docking site for the protease in the trapped acyl-intermediate complex following protease translocation. Ser-365 thus lies at the distal end of strand 5 of the A-sheet and Ile-207 resides on the loop between helix F and strand 3A that overlays the distal ends of strand 5A and the inserted RCL (strand 4A) (Fig. 1). These residues correspond to those that lie directly in the interface with protease (Ser-365) or those that reside close to the protease interface and directly in the helix F–sheet A interface (Ile-207) in structures of the final inhibited serpin–protease complex (7, 24, 25). Because changes in both interfaces on the route to the final serpin–protease complex are coupled (26), the mutation of these residues could be expected to affect the stability of the final complex and could in part explain the greatly enhanced stoichiometries of thrombin and factor Xa inhibition by these variants. The finding that the I207T variant inhibits proteases with modestly enhanced inhibition stoichiometries suggests that the threonine substitution minimally disrupts the interfaces, possibly because of the comparable size of the threonine and isoleucine side chains, whereas the alanine substitution creates a cavity in the helix F–sheet A interface that destabilizes the complex. Because Ser-365 lies directly in the serpin–protease interface, the S365L mutation may destabilize the antithrombin–protease complexes more drastically than the Ile-207 mutations, accounting for the exclusive reaction of this variant as a substrate of thrombin and factor Xa. However, this hypothesis could not be directly demonstrated because only cleaved serpin was observed without any detectable serpin–protease complex by SDS-PAGE in reactions of this variant with protease.

The Ile-207 and Ser-365 mutations also locate close to the cleaved RCL that is inserted as strand 4 of the A-sheet in the final serpin–protease complex. The mutations could thus additionally enhance the partitioning of the acyl-intermediate toward the substrate pathway by slowing the rate of RCL insertion and allowing a greater fraction of the acyl-intermediate to deacylate before the complex is stabilized. The finding that the I207T mutation reduced the apparent rate of protease inhibition but that this rate was nearly normalized to the wild-type rate after correction for the increased inhibition stoichiometry supports this alternative explanation for the effect of this
mutant on acyl-intermediate partitioning. The modest defect observed in protease inhibition assays of this mutant accounts for the reduced anticoagulant activity observed in assays of the patient plasma. The greater enhancement of substrate reactivity of the I207A mutant could in part also be caused by this mutation slowing the rate of RCL insertion into the A-sheet. This is supported by the normalization of the reduced rate of thrombin inhibition of this mutant to the wild-type rate after correcting for the increased inhibition stoichiometry. However, a similar correction of the factor Xa inhibition rate gave an anomalously higher rate than wild type, implying that complex instability also contributes to the enhanced substrate reactivity. Interestingly, in both Ile-207 mutants, the substrate reaction was increased more in the heparin-activated inhibitor than in the free or inactivated inhibitor, and was greatest in reactions with factor Xa than with thrombin. These findings are consistent with previous findings that heparin activation enhances exosite interactions between protease and the serpin in the acyl-intermediate complex that slow the rate of RCL insertion into the central A-sheet (6). They are also in keeping with prior studies that have shown that the rate of deacylation of antithrombin–factor Xa complexes competes with RCL insertion and protease translocation better than antithrombin–thrombin complexes (2). This accounts for the more pronounced SI effects on mutant antithrombin reactions with factor Xa than with thrombin.

Recent FRET and fluorescence perturbation studies have suggested that the insertion of the serpin RCL into the central β-sheet after acylation/cleavage, as well as the concomitant translocation of protease to its final docking site, involves a two-step process: an initial rapid insertion of the RCL that causes the protease to be translocated close to strand 2 at the distal end of the A-sheet, followed by a very slow conformational change involving changes in helix F–sheet A interactions that shift the protease closer to strand 5 of sheet A and in contact with the residues mutated in this study (26). The initial translocation partly inactivates protease by stripping the RCL and P1 residue from the protease active site, whereas the subsequent protease movement further distorts the protease and stabilizes the complex. In the context of this model, our findings are consistent with the mutations affecting both steps involved in translocating the protease and stabilizing the final serpin–protease complex.

The functional consequences of these mutations in the deficiencies of antithrombin identified in the carriers could also be due to other mechanisms apart from the inhibition defects. The stressed native conformation of serpins, required for an efficient inhibitory mechanism, makes serpins vulnerable to environmental or genetic factors leading to aberrant conformation (7, 21, 27, 28). Both Ser-365 and Ile-207 residues are highly conserved among antithrombins of different species, which implies that they have structural relevance (28). This could explain the observations that the S365L mutant forms disulfide-linked dimers and precipitates during concentration. Similarly, the I207T and I207A mutations were found to exhibit lower melting temperatures than wild type, indicative of a reduced structural stability. The reduced native state stability of these variants could explain the modest increase in heparin affinities if the instability was partly relieved in activated states with higher heparin affinity.

In conclusion, we have identified a new functional domain of antithrombin that is critical for the efficient trapping of proteases in stable acyl-intermediate complexes. Ser-365 and Ile-207 residues reside in or on the periphery of the interface of the final docking site of the protease with the serpin and thereby may contribute to the stability of the final complex. These residues also lie adjacent to the inserted RCL and may thus affect the rate at which the RCL is inserted into the A-sheet. Mutations of these residues decrease antithrombin reactivity by enhancing the reaction of antithrombin as a substrate. Our data further suggest relevant interactions between residues located in this region and target proteases in different serpins during the inhibition process. Most interestingly, Ile-207 is a likely homologue of Leu-176 in α1-protease inhibitor, and is found in the serpins, squamous cell carcinoma antigen-1 and 2, and megisin (29). Alanines close to the corresponding residues of Ile-207 and Ser-365 in α1-protease inhibitor (Ala-183 and Ala-332) have been shown to be important in the accommodation of the protease in the serpin–protease complex, supporting a role for Ile-207 and Ser-365 in complex stability. Therefore, the effect of the homologous residues of Ser-365 and Ile-207 in other serpins should be evaluated in future studies.

**New functional domain in antithrombin**

**Experimental procedures**

**Functional and genetic analysis of patients and family members**

During the last 10 years, our group has recruited 142 unrelated patients with antithrombin deficiency, mainly from different Spanish hospitals. Characterization of antithrombin deficiency was done as described elsewhere (19, 20). Briefly, (i) plasma antithrombin activity (anti-FXa and anti-FIIa) was determined by chromogenic assays in the presence of heparin; (ii) antigen levels were determined by immunodiffusion and ELISA; (iii) heparin affinity of plasma antithrombin was evaluated by crossed-immunoelectrophoresis; (iv) electrophoretic features of plasma antithrombin were assayed using native-PAGE (in the presence and absence of 6 M urea) and SDS-PAGE under reducing and nonreducing conditions; and (v) molecular analysis of SERPINC1, the gene encoding antithrombin, was performed by PCR amplification and sequencing. These studies included sequencing of the promoter region (1500 bp) and the seven exons and flanking regions. Additionally, gross gene defects were evaluated by multiplex ligation-dependent probe amplification (MLPA).

**Recombinant expression and purification of wild type and antithrombin variants**

**Site-directed mutagenesis**—Site-directed mutagenesis was carried out using the Stratagene QuikChange Site-Directed Mutagenesis kit (Agilent, Madrid, Spain) or Quick Exchange kit (Invitrogen) and the appropriate primers. S365L, I207T, and I207A mutations were confirmed by DNA sequencing.

**HEK-EBNA cell expression**—We used the pCEP4-AT plasmid containing the cDNA sequence of human antithrombin, generously provided by Prof. J. Huntington (Cambridge Institute for Medical Research (CIMR), Cambridge, UK). The plasm-
mid, commonly used to produce recombinant antithrombin in human embryonic kidney cells expressing the Epstein-Barr nuclear antigen 1 (HEK-EBNA), had the S137A mutation to only produce β-antithrombin that lacks glycosylation at Asn-135 (30). HEK-EBNA cells were grown and transfected with the plasmid containing the mutation as has been described elsewhere (31), and conditioned medium was harvested and stored at −80 °C.

**Insect cell expression**—Recombinant antithrombin variants in this case were constructed on an N135Q background to block glycosylation of Asn-135 and produce β-antithrombin (32). Variants were produced in baculovirus-infected insect cells using the expression system from Invitrogen, as described by the manufacturer. Recombinant antithrombins were purified on a 5-ml Hi-Trap heparin-Sepharose column followed by ion-exchange chromatography using a mono Q column, as described previously (33, 34). Those fractions containing pure antithrombin were finally desalted, and concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 37,700 M⁻¹ cm⁻¹ (35). The purity of proteins was evaluated by 10% SDS-PAGE and Coomassie Blue staining. Finally, proteins were stored at −80 °C.

**Formation of covalent complexes**

Recombinant antithrombin variants secreted to the conditioned medium of HEK-EBNA cells were incubated with FXa (8 μM) or FIIa (19 μM) (Enzyme Research Laboratories, Swansea, UK) for 15 min at 37 °C. The reaction was carried out with and without 6.6 μM unfractionated heparin (Mayne, Madrid, Spain) for 10 min. The final reactions were analyzed by SDS-PAGE under nonreducing conditions. Antithrombin was immunostained with rabbit anti-human antithrombin polyclonal antibody (Sigma-Aldrich, Madrid, Spain), followed by donkey anti-rabbit IgG-horseradish peroxidase conjugate (GE Healthcare, Barcelona, Spain), with detection via an ECL kit (GE Healthcare).

**Assay conditions**

Antithrombin–protease reaction stoichiometries and kinetics were measured at 25 °C in 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4, buffer for reactions with thrombin and factor Xa. The ionic strength of the buffer was 0.15.

**Stoichiometry of antithrombin–protease reactions**

The stoichiometries for the reactions of antithrombin purified variants with FIIa and FXa were determined as described previously (36). Briefly, increasing concentrations of inhibitor were added to a fixed concentration of 100 nM protease, giving molar ratios of inhibitor to protease of up to 3 for I207T and 40 for I207A in a final volume of 100 μl. Reactions were carried out both in the absence and in the presence of full-length heparin or pentasaccharide, which was fixed at an equimolar concentration with the protease. After incubating for times sufficient to complete the reaction (95%), 5 μl of the reaction mixture was added to 1 ml of substrate (100 μM S-2238 for FIIa or 100 μM Spectrozyme FXa for FXa), and the residual enzymatic activity was measured from the initial linear rate of change of absorbance at 405 nm. The decrease in protease activity with increasing molar ratio of inhibitor/protease was fit by linear regression to obtain the stoichiometry from the abscissa intercept.

**Kinetics of antithrombin–protease reactions**

Association rate constants for reactions of recombinant antithrombin variants with target proteases in the absence or presence of pentasaccharide or full-length heparin were measured under pseudo-first-order conditions by using between 10 and 80-fold molar excess of inhibitor over protease as in previous studies (5, 34). For reactions with all proteases in the absence of heparin and those reactions with proteases in the presence of pentasaccharide whose rate constants were 10⁴ M⁻¹ s⁻¹ or less (or in some cases as high as 10⁵ M⁻¹ s⁻¹), full reaction time courses of the loss of enzyme activity were obtained and fit by a single exponential function with a zero activity end point. Pentasaccharide or heparin was present in these full time course experiments at levels that saturated antithrombin. Assays of residual enzyme activity were conducted by removing aliquots of the reaction at different times, diluting into appropriate chromogenic substrates, and measuring the initial rate of substrate hydrolysis as in previous studies (37). Association rate constants for free antithrombin or antithrombin–pentasaccharide/heparin complex reactions were obtained from fitted exponential rate constants by dividing by the concentration of antithrombin. For all other reactions in the presence of pentasaccharide or full-length heparins, reactions were done for a fixed time as a function of the concentration of heparin, and the loss in enzyme activity was fit by a single exponential function with heparin concentration instead of time as the independent variable (34). The association rate constant for the reaction of the antithrombin–heparin complex was obtained from the fitted exponential rate constant by dividing by the fixed reaction time and by a factor that corrected for the fraction of heparin that was bound by antithrombin.

**Determination of denaturing temperature**

Thermal denaturation of antithrombin variants was monitored by changes in tryptophan fluorescence, with excitation wavelength at 280 nm and emission wavelength at 345 nm using the temperature-controlled SLM Instruments fluorometer. The rate of temperature change of 0.5 °C/min was used (11). Antithrombin concentration was 100 nm in phosphate buffer pH 7.4 and I = 0.15. The data were fitted to the Van’t Hoff equation

\[
\Delta G = -RT \ln (F_{\text{obs}} - F_n) / (F_d - F_{\text{obs}}) = \Delta H_m (1 - T / T_m)
\]

(Eq 1)

where \( \Delta G \) = Gibb’s free energy; \( R \) = gas constant; \( T \) = absolute temperature; \( F_{\text{obs}} \) = fluorescence observed; \( F_{\text{nt}} \) = fluorescence coefficient of the native state; \( F_d \) = fluorescence coefficient of the denatured state; \( \Delta H_m \) = enthalpy of denaturation. Fluorescence coefficients were determined from the linear parts of plots.

**Heparin binding to antithrombin variants**

Equilibrium binding of pentasaccharide to purified recombinant variants was evaluated by titrating pentasaccharide and
monitoring the tryptophan fluorescence enhancement, as described previously (26). Because of the tight binding of recombinant antithrombin to heparin at physiologic ionic strength, binding studies were conducted in the phosphate buffer used for studying antithrombin–pentasaccharide reactions, but at a higher ionic strength (I = 0.35, attained by adjusting the NaCl concentration to 0.3 M) to allow more accurate measurements of \( K_D \). Fluorescence titrations were computer-fit by the quadratic equilibrium-binding equation assuming a 1:1 binding stoichiometry to determine values for \( K_D \) and the maximal fluorescence change (36).

Author contributions—S. A., G. I., and S. T. O. performed experiments, analyzed and interpreted data, and wrote the manuscript. S. T. O., J. C., V. V., and I. M. M. designed and directed experiments and revised the manuscript.

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Note added in proof—In the version of this article that was published between different sections of a gel. This error has now been corrected.

References


New functional domain in antithrombin


Disease-causing mutations in the serpin antithrombin reveal a key domain critical for inhibiting protease activities
Sonia Águila, Gonzalo Izaguirre, Irene Martínez-Martínez, Vicente Vicente, Steven T. Olson and Javier Corral

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