The Serpin Inhibitory Mechanism Is Critically Dependent on the Length of the Reactive Center Loop*

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The recent crystallographic structure of a serpin-protease complex revealed that protease inactivation results from a disruption of the catalytic site architecture caused by the displacement of the catalytic serine. We hypothesize that inhibition depends on the length of the N-terminal portion of the reactive center loop, to which the active serine is covalently attached. To test this, α1-antitrypsin Pittsburgh variants were prepared with lengthened and shortened reactive center loops. The rates of inhibition of factor Xa and of complex dissociation were measured. The addition of one residue reduced the stability of the complex more than 200,000-fold, and the addition of two residues reduced it by more than 1,000,000-fold, whereas the deletion of one or two residues lowered the efficiency of inhibition and increased the stability of the complex (2-fold). The deletion of more than two residues completely converted the serpin into a substrate. Similar results were obtained for the α1-antitrypsin variants with thrombin and for PAI-1 and PAI-2 with their common target tissue plasminogen activator. We conclude that the length of the serpin reactive center loop is critical for its mechanism of inhibition and is precisely regulated to balance the efficiency of inhibition and stability of the final complex.

Serpins are the predominant protease inhibitors in mammals and are involved in the regulation of key steps of tightly regulated proteolytic cascades such as blood coagulation, fibrinolysis, and complement activation (1, 2). Their relative bulk compared with the inhibitory domains of the 19 other protein families of serine protease inhibitors (3) provides the serpins with potential regulatory sites, but it is the unique mechanism of inhibition that accounts for the evolutionary success of the serpins. Serpins inhibit proteases by subverting the proteolytic cycle after the formation of the acyl-enzyme intermediate. This is achieved by preventing hydrolysis of the ester bond between the carbonyl carbon of the reactive center P1 residue and the Oγ of the active site serine. Inhibition by serpins is best described as a suicide-substrate mechanism (4, 5), as shown in Scheme 1.

The kinetic model of the reaction of a serpin, I1, with a protease, P, is identical to that of the proteolysis of a substrate. After formation of a Michaelis complex, [IP], the carbonyl carbon of the reactive center P1 residue undergoes nucleophilic attack by the catalytic serine Oγ (Ser-195). The attack proceeds through a tetrahedral transition state that requires a precise geometry of main-chain amide groups on residues 193 and 195 of the protease for continuation to the acyl-enzyme intermediate, [IP]*. At this stage, there is an ester bond between the Ser-195 Oγ and the carbonyl carbon of the P1 residue, and the peptide bond between P1 and P1′ has been broken. The serpin then undergoes a dramatic conformational change of incorporation of the entire reactive center loop as the fourth strand in β-sheet A, resulting in a 70-Å translocation of the attached protease to the opposite pole of the serpin (Fig. 1a). The inhibited serpin-protease complex, IP*, is stable for weeks to years depending on the serpin-protease pair and conditions but eventually will dissociate via k5 to yield the products of normal proteolysis, cleaved serpin, I*, and active protease, P. If loop insertion is not rapid enough to compete with decayation, then the reaction proceeds directly to the cleaved product by a step defined by k6. The relative rates of k4 and k5 determine the stoichiometry of inhibition. Serpins have also been demonstrated to induce proteolytic susceptibility in inhibited proteases, resulting in a dead-end pathway, k6, for the protease (6–8).

The recent structure of a serpin-protease complex shows how inhibition results from a conformational displacement and deformation of the protease (9) (Fig. 1a). The deformation is brought about by the overlap of the hyperstable serpin and the destabilized protease. A principal contribution to the loss of stability in the protease is the distortion of its active site, which is caused by the plucking of the active site serine away from its catalytic partners (Fig. 1b). This distortion of the active site disrupts the interactions in the protease that were formed to stabilize it at the stage ofzymogen activation (10). More critically, the displacement of the serine from the catalytic site of the protease effectively ensures the kinetic stability of serpin-protease complex by preventing hydrolysis of its ester bond with the serpin through destruction of the oxyanion hole (Fig. 1b) (9). It is apparent from the structure of the complex that a critical factor in producing the distortion of the protease is the limited length of the fully inserted reactive center loop of the serpin. This tightness of the tethering of the ester bond and the leverage provided through the overlap of the serpin and the protease effectively “plucks” the serine from its catalytic site. Supporting evidence for the critical contribution of this tight length of the reactive center loop to the inhibition rate of the protease can be derived from the results reported here and previously (9).
The Serpin Mechanism Depends on Reactive Center Loop Length

linkage is given by the observation of a consistently conserved reactive center loop length in the inhibitory serpins. The loop in almost all the serpins is formed by 17 residues, from the glutamate (P17) at the base of the proximal hinge of the loop to the reactive center (P1) residue (11). This is not invariant, but no functional inhibitory serpin has more than 17 residues in the reactive center loop, and only the α2-antiplasmin (12), C1-inhibitor (13), and CrmA (14, 15) have 16-residue loops. The only example a serpin, natural or recombinant, with an 18-residue reactive center loop was for a variant of α2-antiplasmin, which was identified from a patient with a bleeding disorder (16).

Here we assess the effect of a systematic change in loop length, N-terminal to the reactive center, on the inhibitory activity of the recombinant Pittsburgh, P1 Arg, variant of α1-antitrypsin (17). Modified versions of the Pittsburgh α1-antitrypsin, with reactive center loop deletions of 1–4 residues and insertions of one and two residues were prepared (Fig. 2), and their ability to form stable complexes with factor Xa and thrombin were examined. PAI-1 and PAI-2 variants with an extra residue in the reactive center loop were also prepared, and complex formation with their target protease tPA was assessed. The results define the critical requirements for effective inhibition and also clearly demonstrate the way in which the deformation mechanism provides the serpins with the selective advantage of stable and irreversible inhibition.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, and oligonucleotides were synthesized by Life Technologies, Inc. The expression vector pQE31 and nickel-nitrilotriacetic acid gel were from Qiagen. Isopropyl-β-D-thiogalactopyranoside was from Melford Laboratories Ltd. (Suffolk, England). Kanamycin sulfate was from Roche Molecular Biochemicals. Hitrap Q-Sepharose columns were from Amersham Pharmacia Biotech. Ampicillin, bovine thrombin, and bovine factor Xa were from Sigma. Protease concentrations were determined by active site titrations. The substrates S-2222 and S-2238 were from Chromogenix. Phe-Pro-Arg-chloromethylketone and the tissue plasminogen activator tPA were from Calbiochem (CN Biosciences UK, Nottingham, United Kingdom). All experiments were conducted at room temperature (22 ± 1 °C).

Construction and Mutagenesis of Expression Plasmids—Human α1-antitrypsin cDNA was amplified by polymerase chain reaction and inserted into the expression vector pQE31 by the restriction sites BamHI and HindIII. Mutagenesis was carried out by a two-step polymerase chain reaction. The length of the reactive center loop was altered as shown in Fig. 2. Each variant of α1-antitrypsin is denoted according to change in the reactive center loop length as Add-1 and Add-2 or Del-1, -2, -3 or -4. All mutations were confirmed by DNA sequencing. The recombinant α1-antitrypsin was expressed with an MrSHHIIIHIH tag at the N terminus, which can bind to nickel-nitrilotriacetic acid agarose. Human wild type and Add-1, PAI-1, and PAI-2 were similarly prepared, and the expression and purification were performed as described previously (18, 19).

Expression and Purification of Recombinant Protein—Recombinant α1-antitrypsin was purified from the soluble fraction of Escherichia coli lysate. Briefly, expression plasmids were transformed into S113009 (pREP4) cells and grown in 2 liters of 2TY at 37 °C until A600 = 0.8–1.0; then isopropyl-β-D-thiogalactopyranoside was added to a concentration of 1 mM, and the culture was transferred to 30 °C for a further 3 h. The cells were collected by centrifugation, resuspended in buffer A (10 mM phosphate buffer, pH 8.0, 0.5 M NaCl, and 1 mM β-mercaptoethanol), and disrupted by sonication. The supernatant of the cell lysate was loaded onto a nickel-nitrilotriacetic acid column (20-ml bed column), and after washing to baseline with buffer A, the bound protein eluted as a shoulder peak with an imidazole gradient (0–0.2 M). The fractions were collected, dialyzed against buffer B (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 1 mM β-mercaptoethanol) and loaded onto a Hitrap-Q-Sepharose column (5 ml). The column was then washed with a NaCl gradient (0–0.5 M) in buffer B. α1-Antitrypsin was eluted as the major peak (second peak) around 0.2 M NaCl. The fractions were pooled, and protein concentrations were determined spectrophotometrically using an extinction coefficient of 27,560 M⁻¹ cm⁻¹ (20). The samples were snap-frozen in liquid nitrogen and stored at −70 °C. All α1-antitrypsin variants were confirmed to be in pure monomeric form by SDS and native PAGE.

Complex Formation by SDS-PAGE—α1-Antitrypsin was mixed with protease (3:1 molar ratio) at room temperature (22 ± 1 °C). Samples were taken at timed intervals, mixed immediately with reduced SDS-loading buffer at 100 °C, and heated for a further 3 min. SDS-gel electrophoresis (21) was performed in a 12% gel, and the protein was visualized by Coomassie Blue or silver staining.

Stoichiometries of Inhibition—Stoichiometries of inhibition were determined by incubating increasing concentrations of α1-antitrypsin with trypsin (18, 19). Stoichiometries of inhibition were determined by increasing concentrations of α1-antitrypsin with trypsin (3:1 molar ratio) at room temperature (22 ± 1 °C) and incubated from 30 min to 4 h in PBS with 0.1% PEG 8000. The residual amidolytic activity was determined by the addition of 0.1 mM S-2222 or S-2238 substrate. Linear regression analysis of the decrease in protease activity with an increasing concentration of α1-antitrypsin yielded the estimates for the stoichiometry of inhibition as the intercept on the abscissa. The stoichiometry of inhibition for Add-1 and Add-2 α1-antitrypsin was esti-

Fig. 1. Formation of the serpin-protease complex (9). a, a ribbon depiction of α1-antitrypsin with trypsin (cyan and red) aligned above it (left) and of the formed complex (right) showing the full incorporation of the cleaved reactive center loop (yellow) as the middle strand of the A-sheet (blue). The displacement of the protease causes a disruption of 37% of its ordered structure (red). The position of the reactive center loop additions and deletions studied here is indicated by the red arrow. b, the effect of complex formation on the loop containing the active serine (Ser-195) of trypsin is demonstrated by comparing native trypsin (magenta) on the left, with trypsin in complex (green) with α1-antitrypsin (yellow) on the right. The reactive center loop of α1-antitrypsin from P3 to P1 (Met-358) is in yellow and shows the ester bond between catalytic serine Oγ (Ser-195) and the P1 carbonyl carbon. The extension of the active site loop results from the plucking of Ser-195 from its original position through its ester linkage to Met-358.
mated from an SDS gel by comparing the band density of cleaved and complexed α1-antitrypsin at an early time point before significant cleaved material accumulated as a result of complex dissociation. Band densities were standardized by dividing by the molecular weight of cleaved and complexed species.

Rates of Protease Inhibition—The rates of inhibition of factor Xa and thrombin by recombinant α1-antitrypsin variants were determined at room temperature (22 ± 1°C) by a discontinuous assay procedure. Briefly, under pseudo first-order conditions, 10 μl of 2 μM or 0.2 μM α1-antitrypsin variants were mixed with 10 μl of 50 nM or 10 nM protease in PBS with 0.1% PEG 8000. The residual protease activity was determined at timed intervals by diluting the reaction mixture into the assay buffer containing 0.1 mM S-2222 or S-2323. The observed rate constant, \( k_{\text{obs}} \), for the reaction was obtained from the slope of a semilog plot of the residual protease activity against time, and the second-order rate constant, \( k_{\text{app}} \), was calculated by dividing \( k_{\text{obs}} \) by the initial α1-antitrypsin concentration.

Rates of Complex Dissociation—Rates of complex dissociation were determined essentially as described previously (22, 23). α1-Antitrypsin complexes with factor Xa or thrombin were prepared by incubating enzyme with a 5–10-fold molar excess of α1-antitrypsin for 2–240 min and subsequently diluted 500-fold into 0.2 mM substrate S-2222 or S-2233 to monitor complex dissociation. High substrate concentrations were used to ensure that the dissociated protease would not react with the excess α1-antitrypsin. The reappearance of proteolytic activity, reflecting complex dissociation, was monitored continuously for 10–150 min by measuring change in A at 405 nm at room temperature (22 ± 1°C). Under these experimental conditions, less than 5% of substrate was converted to product, and less than 3% of the complex was dissociated, so that an initial rate of complex dissociation was measured. Data were fit satisfactorily to Equation 1, which describes the initial rate of enzyme generation (22),

\[
A_t = A_0 + V_0 \times t + k_{\text{off}} \times \frac{[E - I]_0}{TN} \times t^2/2 \tag{Eq. 1}
\]

where \( A_t \) and \( A_0 \) are the absorbance at time \( t \) and time zero, respectively, \( V_0 \) is the rate of change in \( A_{\text{loss}} \) at time zero, \( k_{\text{off}} \) is the first-order rate constant for complex dissociation, \( [E - I]_0 \) is the starting concentration of complex, and \( TN \) is the turnover number for the hydrolysis of substrate by enzyme under the conditions of the experiment and was independently measured. Complex dissociation is irreversible because serpin is released from the complex in an inactive cleaved form rather than in the native intact form (24). The dissociation rate of the α1-antitrypsin Add-2-factor Xa complex was too fast to be analyzed by the above method and was therefore determined by SDS-PAGE. Briefly, Add-2 was mixed with factor Xa at a molar ratio of 2.5:1 at room temperature (22 ± 1°C) for 1 min, and then 1 mM Phe-Pro-Arg-chloromethylketone was added to neutralize the free factor Xa and stop the association of factor Xa with excess inhibitor. Time points were taken soon after and the samples were mixed immediately with reducing SDS-loading buffer at 100°C and heated for a further 3 min. SDS-gel electrophoresis was performed as described above. The density of the complex bands was determined by scanning and using the software Quantity One (Bio-Rad) and analyzed by a semilog plot against dissociation time. The method was validated using the Add-1 mutant, in which comparable rates were obtained using both the chromogenic and the SDS-PAGE methods (see Table I and Fig. 4).

RESULTS

**SDS-PAGE of Serpin-Factor Xa Reactions**—The insertion and deletion variants were created on the α1-antitrypsin Pittsburgh (P1 Arg) (17) background to ensure that the changes in the reactive center loop length would not affect the site of proteolytic attack by thrombin and factor Xa. The normal targets of wild-type α1-antitrypsin, trypsin, chymotrypsin, and elastase react with less specificity for the P1 residue, and thus changes in reactive center loop composition might affect the site of proteolytic attack, as has been observed previously (25, 26). The insertions and deletions were constructed as shown in Fig. 2 and were placed between P3 and P4 so as not to disrupt the rate or thermodynamics of reactive center loop insertion. Because of the complexity of the serpin-protease reaction mechanism (Scheme 1), reactants and reaction products are best visualized as bands on an SDS gel. Fig. 3 clearly illustrates the effects of altering the length of the serpin reactive center loop on the initial formation and eventual dissociation of the serpin-protease complex. The first gel (Pitts) confirms that the unmodified α1-antitrypsin Pittsburgh is an effective inhibitor of factor Xa, with rapid formation of complex and a minor amount of cleaved form. The complex is stable over the 60 min of incubation. When three residues are deleted from the reactive center loop (Del-3), no complex is observed, and all of the α1-antitrypsin is cleaved.

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**TABLE I**

| Complex          | Pitts-Xa | Add-1-Xa | Add-2-Xa | Del-1-Xa | Del-2-Xa | Pitts-IIa | Add-1-IIa | Del-1-IIa |
|------------------|----------|----------|----------|----------|----------|-----------|-----------|-----------|
| \( k_{\text{app}} \) (s\(^{-1}\)) | 3.2 × 10\(^4\) | 1.6 × 10\(^4\) | ND       | 1.0 × 10\(^3\) | 3.0 × 10\(^3\) | 1.8 × 10\(^5\) | 4.1 × 10\(^5\) | 5.0 × 10\(^7\) |
| SI               | 1.2      | 1.2\(^a\) | 1.2\(^a\) | 1.2\(^a\) | 1.2\(^a\) | 1.2\(^a\) | 1.4\(^a\) | 3.1\(^a\) |
| \( k_{\text{off}} \) (s\(^{-1}\)) | 8.4 × 10\(^{-9}\) | 1.8 × 10\(^{-3}\) | 1.9 × 10\(^{-3}\) | 1.3 × 10\(^{-2}\) | 4.5 × 10\(^{-9}\) | 4.8 × 10\(^{-9}\) | 8.4 × 10\(^{-8}\) | 2.0 × 10\(^{-5}\) |
| \( t_{1/2} \)     | 136 weeks | 6 min    | ND       | 53 s     | 255 weeks | 239 weeks | 14 weeks | 10 h      |

\(^a\) Determined from densitometry of SDS gels as described under “Experimental Procedures.”

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**FIG. 2.** Reactive center loop sequences of α1-antitrypsin Pittsburgh (a), PAI-1 (b), and PAI-2 (c) variants showing the positions of additions and deletions in each of the engineered recombinants. The P notation is that of Schechter and Berger (28), wt, wild type.
The Serpin Mechanism Depends on Reactive Center Loop Length

The effect of the addition of one and two extra residues to the reactive center loop of α1-antitrypsin Pittsburgh, shown by gels Add-1 and Add-2, respectively, is dramatic. Whereas in the control gel the α1-antitrypsin-factor Xa complex is stable over the duration of the experiment, the addition of one residue (Add-1) to the reactive center loop results in complete dissociation of the complex by 20 min (lane 9), despite the 2.5-fold excess of inhibitor over protease. The dissociation is clearly via deaclylation, because the only two reaction products are cleaved serpin and intact active protease. The excess of serpin makes determination of the half-life difficult under these conditions, but it can be estimated at 5 min, because at 5 min (lane 7) most of the native serpin has already reacted, by 10 min (lane 8) half of the complex has dissociated, and by 20 min (lane 9) no complex remains. The addition of two residues to the reactive center loop (Add-2) results in a rate of dissociation that competes with the rate of association, so that by the time all of the native α1-antitrypsin has reacted no complex is detectable. It is not possible to estimate a half-life from this gel, but an upper limit of 1 min can be set, because between the 2- and 5-min time points (lanes 6 and 7) all of the complex has dissociated. It is interesting to note that although in all lanes for Add-1 and Add-2 there is a clear band for free factor Xa, there is no evidence for cleaved complex as in Pitts, Del-1, and Del-2. This may be because of the short half-life of the complex, but may also reflect the loss of induced proteolytic susceptibility of proteases within the complexes with reactive center loop lengthened α1-antitrypsin.

Stoichiometries and Rates of Inhibition of Factor Xa—Stoichiometries and rates of factor Xa inhibition by the control Pittsburgh α1-antitrypsin and the variants Del-1, Del-2, Add-1, and Add-2 are given in Table I. It is clear from Fig. 3 that the stoichiometry of inhibition is extremely sensitive to truncation of the reactive center loop of α1-antitrypsin. In the extreme case of three or more deletions, α1-antitrypsin is effectively rendered a substrate to factor Xa, and in the case of two deletions, there is significantly more cleaved material visible on the gel. The stoichiometry of inhibition in solution for Del-1 is indistinguishable from the control, but for Del-2 the stoichiometry of inhibition is nearly three, indicating that for each reaction resulting in a stable complex, two reactions do not. In the case of the loop insertion variants, it is possible that the large amount of cleaved α1-antitrypsin evident on the gels is caused by a change in stoichiometry of inhibition, an increase in the rate of dissociation, or both. Cleaved serpin is the final product of two distinct pathways defined by the rate constants $k_3$ and $k_5$ (Scheme 1). The stoichiometry of inhibition is defined as $(k_3 + k_4)/k_4$ and is thus independent of the complex dissociation term, $k_5$; however the rate of dissociation of the complex depends on the concentration of the complex. This rendered determination of the stoichiometries using the traditional chromogenic method problematic, because the reactions need to proceed to completion. It was possible to estimate stoichiometries at early time points from SDS gels by dividing the band densities for the cleaved and complex products by their respective molecular weights. The stoichiometries of inhibition for Add-1 and Add-2 thus were determined to be indistinguishable from the control (Table I). Thus, it is possible to delete one residue and add up to two without significantly affecting the relative rates of $k_3$ and $k_4$, and deletion of more than two may significantly alter the final conformation of the complex such that full protease translocation can not be achieved.

Association rates are also given in Table I. The rate of inactivation of factor Xa by α1-antitrypsin is only mildly sensitive to the length of the reactive center loop, because both the insertion of a single residue and the deletion of up to two residues result in a similar small decrease in second-order rate constant (2- to 4-fold) when accounting for changes in stoichiometry of inhibition.

Rates of Dissociation of the α1-Antitrypsin-Factor Xa Complexes—Fig. 3 unequivocally demonstrates the major effect of increasing the length of the serpin reactive center loop: the reduction of stability of the final serpin-protease complex. This is predicted from the crystallographic structure of the α1-antitrypsin-trypsin complex, in which the disruption of the catalytic architecture is the direct result of a 3.5 Å shift in position of the catalytic serine. An increase in the number of reactive center loop residues by one would be equivalent to adding 3.5 Å to its length. The rates of complex dissociation are given in Table I. The insertion of one extra residue in the reactive center loop decreased the stability of the complex by 200,000-fold, and the addition of two residues destabilized it by another 5-fold. Fig. 4 shows representative data and fits for the determination of first-order rate constants for complex dissociation. The figure illustrates the determination of dissociation rates for the complex between Add-1 α1-antitrypsin and factor Xa by the chromogenic (Fig. 4a) and SDS-gel densitometry (Fig. 4b) methods. The fact that the rates are indistinguishable validates the SDS-gel method for determination of the rate of dissociation of the Add-2 α1-antitrypsin-factor Xa complex (Fig. 4c), for which...
The Serpin Mechanism Depends on Reactive Center Loop Length

The tight conservation of the length of serpin reactive center loops is striking when compared with the wide variance in the length of surface loops for other protein families. Indeed, one might predict that the length of the reactive center loop would vary significantly between members of the serpin family because it is this region that determines protease specificity. Conservation of loop length, hinge region (P15–P8) composition, and an absence of charged or bulky even-registered P residues indicates that the entirety of the reactive center loop is involved in the serpin mechanism of inhibition. This conclusion is supported by the only structure of a serpin-protease complex solved to date: that of the prototypical serpin α1-antitrypsin trypsin (9). The structure revealed that the limited length of the reactive center loop induced a 3.5-A displacement of the catalytic serine from the active site through full reactive center loop incorporation into β-sheet A at the acyl-enzyme intermediate step of the proteolytic cycle. This plucking of the active site serine away from its catalytic partners is only possible if leverage is applied through interactions between the body of the serpin and the protease. Such interactions are clear from the structure of the α1-antitrypsin-trypsin complex. On the basis of these observations we hypothesized that the effect of an increase in the length of the reactive center loop would be a reduction in kinetic stability of the covalent serpin-protease complex and furthermore that this would be general to all serpin-protease complexes. We set out to test this hypothesis by creating loop-length variants of Pittsburgh α1-antitrypsin variants Add-1, Add-2, Del-1, and Del-2 with thrombin and for PAI-1 and PAI-2 with their common target, tissue plasminogen activator. For all, there is a clear trend toward loss of complex stability relative to the control caused by an increase in the length of the reactive center loop. Kinetics and stoichiometries of inhibition of thrombin by the α1-antitrypsin variants and rates of complex dissociation are given in Table I. In contrast to the complexes with factor Xa, the addition of one residue (Add-1) more than doubles the rate of complex formation and effects a mere 240-fold reduction in complex stability. The insertion of an additional residue (Add-2) is required to reach the rates of complex dissociation observed for the Add-1 complex with factor Xa. Conversely, the deletion of just one residue from the reactive center loop (Del-1) is sufficient to increase the stoichiometry of inhibition by 3-fold, decrease the rate of association by 3-fold, and increase the stability of the complex by 4-fold. The increase in stoichiometry of inhibition is similar to what was observed for the reaction of the Del-2 variant with factor Xa, in which the Del-1 mutation had little effect.

**DISCUSSION**

The data presented here clearly demonstrate the critical dependence of the serpin mechanism on the length of the peptide loop between the reactive center P1 and the conserved hinge (Glu-342 using the template numbering of Huber and Carrell (11)). It is also interesting to compare the kinetics for the formation and dissociation of the α1-antitrypsin Pittsburgh variants with the two coagulation proteases, factor Xa and
thrombin. Both followed the general trend shared with the other serpin-protease complexes studied here of a decrease in complex stability with loop elongation and an increase in complex stability with loop truncation. Another trend, most evident for the reaction of the $\alpha_1$-antitrypsin variants with thrombin, is the correlation between loop length and rate of complex formation. The major difference for the factor Xa and thrombin complexes was in their respective sensitivities to loop elongation and truncation. The stability of the complex of $\alpha_1$-antitrypsin Add-1 with factor Xa was reduced by more than 5 orders of magnitude, whereas the stability of the Add-1 complex with thrombin was reduced by only 2 orders of magnitude. Conversely, the deletion of one residue in the Del-1 $\alpha_1$-antitrypsin variant had no effect on the stoichiometry of factor Xa inhibition but resulted in a tripling of the stoichiometry of thrombin inhibition. Such an increase in stoichiometry of factor Xa inhibition required the deletion of two residues from the reactive center loop. We believe this can be explained by differences in plucking stress exerted on the protease active site loop in the two wild-type $\alpha_1$-antitrypsin Pittsburgh-protease complexes. Plucking of the active site loop requires an ester bond between the catalytic serine of the protease and the P1 residue of the serpin and clashes between the bottom of the serpin and the face of the protease containing the active site cleft. The structure of thrombin contains several stretches of sequence not found in factor Xa. Of these, the 60 loop and 147 loop are on the face of the protease that would be in contact with the serpin in the final complex (Fig. 6). The difference in bulk is evident from the figure. Models of the complexes were created on the template of the $\alpha_1$-antitrypsin-trypsin complex to determine regions in which the $\alpha_1$-antitrypsin and factor Xa or thrombin would overlap (Fig. 6, red). Serpins with large loops on the pole where proteases sit in the final complex (Fig. 1a, bottom) would be predicted to have a similar effect of increasing the plucking stress on the active site loop of the protease. The serpin mechanism can clearly accommodate shortening but not elongation of the 17-residue reactive center loop. Serpins with 16-residue reactive center loops may share a requirement for the increased complex stability that a fore-shortened loop affords.

In conclusion, the results presented here provide a clear and consistent picture of a single serpin mechanism of protease inhibition that is critically dependent on a limited reactive center loop length to ensure the kinetic stability of the serpin-protease complex.

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