Intrinsic Specificity of the Reactive Site Loop of α1-Antitrypsin, α1-Antichymotrypsin, Antithrombin III, and Protease Nexin I*

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Members of the serpin (serine protease inhibitor) family share a similar backbone structure but expose a variable reactive-site loop, which binds to the catalytic groove of the target protease. Specificity originates in part from the sequence of this loop and also from secondary binding sites that contribute to the inhibitor function. To clarify the intrinsic contribution of the reactive-site loop, α1-antichymotrypsin has been utilized as a scaffold to construct chimeras carrying the loop of antithrombin III, protease nexin I, or α1-antitrypsin. Reactive-site loops not only vary in sequence but also in length; therefore, the length of the reactive-site loop was also varied in the chimeras. The efficacy of the specificity transfer was evaluated by measuring the stoichiometry of inhibition and complement activation; they consist of 400–450 amino acids organized into three β-sheets and eight or nine α-helices connected by surface loops (1). The connection between the A and C β-sheets constitutes the reactive-site loop. Although the precise inhibitory mechanism of serpins remains to be elucidated, all proposed models involve an initial interaction between the reactive-site loop of the serpin and the catalytic groove of the protease. Within the reactive-site loop, a P1 residue2 plays a crucial role in determining serpin specificity (2, 3). However, many serpins have an arginine for P1 residue, yet inhibit different targets; thus other residues modulate the inhibitor function (4–13).

The serpin α1-antichymotrypsin (ACT) is an acute phase plasma serpin (14–16), which shares 45% sequence identity with α1-antitrypsin, 33% with antithrombin III, and 27% with protease nexin I (3, 17–18). ACT has Leu358 for P1 residue and inhibits chymotrypsin, pancreatic elastase, cathepsin G, and mast cell chymase (19–20). ACT does not inhibit trypsin-like enzymes, but single replacement for arginine of Leu358 dramatically alters its specificity: ACT(P1→Arg) inhibits trypsin and thrombin but not the elastase-like enzymes (5). The serpin α1-antitrypsin (also called α1-protease inhibitor; Refs. 2 and 14) inhibits several elastase-, chymotrypsin-, and trypsin-like enzymes, but substitution for arginine of its P1 methionine is still necessary for effective neutralization of thrombin, factor Xa, activated protein C, and urokinase (6, 21, 22). Antithrombin III (14) has a P1 arginine; it inhibits trypsin, thrombin, and factor Xa but not activated protein C (23–25). Finally, protease nexin 1, which also has a P1 arginine, rapidly inactivates thrombin, urokinase, trypsin, and activated protein C (18, 22–23).

To document further the role of the reactive site loop in controlling the specificity, we have prepared and characterized various chimeras, using ACT as a framework to carry the loop of other serpins with overlapping specificities. Results suggest that, in addition to the sequence of the reactive site loop, the specificity of serpins originates from secondary binding sites and conformational constraints.

MATERIALS AND METHODS

Proteases—Human activated protein C was a generous gift from Drs. J. Stenflo and A. Ohlin (University of Lund, Malmo, Sweden). Human leukocyte elastase (elastase) was from Adsabs Inc. (Athens, GA). Bovine pancreatic trypsin (1,1-tosylamido-2-phenylethyl chloromethyl ketone-treated) and bovine chymotrypsin (1-chloro-3-tosylamido-7-amino-2-heptanone-treated) were purchased from Worthington (Lorne Laboratories, Twyford, UK), and human urokinase (low molecular weight) was from Calbiochem (NovoBiochem, Nottingham, UK). Human thrombin and bovine factor Xa were prepared as described previously (26, 27).

ACT Variants—Mutants of ACT were produced and purified essentially according to Rubin et al. (5, 28). Reactive-site loops were exchanged by cassette mutagenesis using the expression vector pACT

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1 The abbreviations used are: serpin, serine protease inhibitor; ACT, recombinant α1-antichymotrypsin; ACT(P1→Arg), ACT where the P1 residue (Leu358) has been replaced by an arginine; ACT(P1→Arg, P2→Gly), ACT(P1→Arg, P2→Gly), ACT(P1→Arg, P1→proline), ACT(P1→Arg, P2→proline), ACT(P1→Arg, P2→proline), ACT mutant having an arginine for P1 residue and a glycine for P2 residue (proline and alanine for P2 residue, respectively); ACT(P1→Arg, P2→Ala), ACT mutant having an arginine for P1 residue and alanine for P2 residue, respectively); ACT(∆Thr166), ACT where residues Thr166, Arg369, and Arg369 (positions P8, P9, and P11, of the reactive-site loop) have been deleted; ACT(∆Thr166), ACT in which the ACT framework carries the reactive-site loop of antithrombin III; ACT(∆Thr166), ACT in which the ACT framework carries the reactive-site loop of α1-antitrypsin; ACT(∆Thr166), ACT in which the ACT framework carries the reactive-site loop of protease nexin 1; elastase, human leukocyte elastase; SI, stoichiometry of inhibition; pNA, p-nitroanilide.

2 Residues within the reactive-site loop are numbered by analogy with substrates as follows: P8→P9→P10→P11→P12→P13→P14, where cleavage would occur at the P7→P8 bond in a substrate.
generously given by Dr. H. Rubin (University of Pennsylvania, Philadelphia, PA), in which two restriction sites (KpnI and MluI) had been engineered within the ACT coding sequence. The engineered KpnI site, between the codons for the P3" and P3" residues, results in the replacement of the alanines of wild-type ACT with glycine and threonine; the MluI site, between the codons for the P9" and P9" residues, exchanges the P9" valine to threonine (28–29). To express chimeras ACT/ATIII, ACT/AT, ACT/PN1, ACT/PN1(des-TIVR), and ACT/PN1 (Table I), oligonucleotides flanked by the appropriate restriction sites and encoding the reactive-site loops of antithrombin III (5'-C-ATG-ACG-TGG-GCT-TGG-CGC-CCA-GAA-AGC-GCC-TGT-AGC-TGG-CGC-TGC-GCT-5'), of c1-antitrypsin (5'-C-ATGC-TTC-CTG-GAA-GTC-ATG-CCC-GGC-TCC-GCA-AGT-GCC-GAG-GGG-GTC-5') and ACT (5'-C-ATG-GTC-TAC-AAG-GAC-CTT-CGA-TAG-GGC-TAC-TCG-TAG-5'), of a1-antitrypsin (5'-C-ATGC-TTC-CTG-GAA-GTC-ATG-CCC-GGC-TCC-GCA-AGT-GCC-GAG-GGG-GTC-5') and ACT (5'-C-ATG-GTC-TAC-AAG-GAC-CTT-CGA-TAG-GGC-TAC-TCG-TAG-5'), of c1-antitrypsin (5'-C-ATG-GTC-TAC-AAG-GAC-CTT-CGA-TAG-GGC-TAC-TCG-TAG-5'), of a1-antitrypsin (5'-C-ATGC-TTC-CTG-GAA-GTC-ATG-CCC-GGC-TCC-GCA-AGT-GCC-GAG-GGG-GTC-5') and ACT (5'-C-ATG-GTC-TAC-AAG-GAC-CTT-CGA-TAG-GGC-TAC-TCG-TAG-5'), of c1-antitrypsin (5'-C-ATG-GTC-TAC-AAG-GAC-CTT-CGA-TAG-GGC-TAC-TCG-TAG-5') were ligated in pACT by standard techniques (31). Chimera ACT(des-TIVR) was constructed (Table I). Intramolecular Control of Serpin’s Specificity— Stoichiometry of inhibition (SI, i.e., the number of moles of serpin required to inhibit 1 mol of protease) was determined as described previously (30), using chymotrypsin and elafin for the chimeras having a P1 leucine or methionine and using trypsin and thrombin for the chimeras having a P1 arginine. The ability of the chimeras to form SDS-stable complexes was examined by incubating the variant (2 μM) with the protease (1 μM) in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, and 0.2% (w/v) poly(ethylene glycol) (M′ 6000 for 30 min at room temperature. The sample was denatured at 65 °C for 10 min in 0.37 M Tris-HCl, pH 8.8, containing 1% SDS (w/v), 10% glycerol (v/v), and 5% β-mercaptoethanol (v/v) and analyzed by SDS-polyacrylamide gel electrophoresis (gradient 10–20% acrylamide). The overall association rate constant for the formation of a protease-serpin complex (k_on) was estimated by analysis of data from progress curve kinetics completed with a large excess of serpin (6 concentrations, between 0.32 and 10 μM) over the enzyme (a single concentration between 0.01 and 1 mM, depending upon the protease). Inhibition reactions were followed for up to 3 h using a Hewlett-Packard diode array spectrophotometer, but only data corresponding to less than 10% substrate hydrolysis were analyzed. Reactions were initiated by addition of the protease at concentrations of the order of 10 μM. The product formation was linear with respect to enzyme concentration and time. A single kinetic order was observed for all reactions (31). A244 (<Glu-Gly-Arg-pNA) was used with urokinase (Km = 90 μM). The above peptides-pNA substrates were purchased from Chromogenix (Molndal, Sweden). Succinyl-Ala-Ala-Pro-Phe-pNA was used with chymotrypsin (Km = 53 μM), and N-methoxy succinyl-Ala-Ala-Pro-Val-pNA was used with elastase (Km = 121 μM); both were purchased from Sigma. Substrate concentrations were determined from their absorbance at 342 nm using a molar extinction coefficient of 8270 cm⁻¹ (32). Activation of the pNA substrate depended whether competition between hydrolysis and inhibition was desired; substrates with low Km values were used to increase the apparent half-life of complex formation, and those with high Km values were used to minimize competition.

**Characterization of the Chimera Specificity**— Stoichiometry of inhibition (SI, i.e., the number of moles of serpin required to inhibit 1 mol of protease) was determined as described previously (30), using chymotrypsin and elastase for the chimeras having a P1 leucine or methionine and using trypsin and thrombin for the chimeras having a P1 arginine. The ability of the chimeras to form SDS-stable complexes was examined by incubating the variant (2 μM) with the protease (1 μM) in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, and 0.2% (w/v) poly(ethylene glycol) (M′ 6000 for 30 min at room temperature. The sample was denatured at 65 °C for 10 min in 0.37 M Tris-HCl, pH 8.8, containing 1% SDS (w/v), 10% glycerol (v/v), and 5% β-mercaptoethanol (v/v) and analyzed by SDS-polyacrylamide gel electrophoresis (gradient 10–20% acrylamide). The overall association rate constant for the formation of a protease-serpin complex (k_on) was estimated by analysis of data from progress curve kinetics completed with a large excess of serpin (6 concentrations, between 0.32 and 10 μM) over the enzyme (a single concentration between 0.01 and 1 mM, depending upon the protease). Inhibition reactions were followed for up to 3 h using a Hewlett-Packard diode array spectrophotometer, but only data corresponding to less than 10% substrate hydrolysis were analyzed. Reactions were initiated by addition of the enzyme at concentrations of the order of 10 μM. The product formation was linear with respect to enzyme concentration and time. A single kinetic order was observed for all reactions (31). A244 (<Glu-Gly-Arg-pNA) was used with urokinase (Km = 90 μM). The above peptides-pNA substrates were purchased from Chromogenix (Molndal, Sweden). Succinyl-Ala-Ala-Pro-Phe-pNA was used with chymotrypsin (Km = 53 μM), and N-methoxy succinyl-Ala-Ala-Pro-Val-pNA was used with elastase (Km = 121 μM); both were purchased from Sigma. Substrate concentrations were determined from their absorbance at 342 nm using a molar extinction coefficient of 8270 cm⁻¹ (32). Activation of the pNA substrate depended whether competition between hydrolysis and inhibition was desired; substrates with low Km values were used to increase the apparent half-life of complex formation, and those with high Km values were used to minimize competition.

We have constructed ACT chimeras in which the length of the reactive-site loop corresponds to that of either the donor or acceptor (ACT/αAT and ACT/PN1 carrying long reactive-site loop, as well as ACT/αAT(des-TIVR) and ACT/PN1(des-TIVR) carrying shorter loop); because the reactive-site loop of antithrombin III is only one amino acid shorter than that of ACT, only the ACT/ATIII chimera was constructed (Table I). The selectivity
and effectiveness of each chimera toward a number of possible targets were evaluated using three criteria: SI value, ability to form an SDS-stable complex, and \( k_{on} \) value.

Chimeras with proline in \( P_2 \) and arginine in \( P_1 \) exhibited SI values higher than 150 with trypsin and lower than 10 with thrombin (Table I). This is consistent with our observation (30) that replacement of the \( P_2 \) leucine of ACT/\( \{P_1\text{-Arg}\} \) with proline causes a dramatic increase in the SI value with trypsin but not with thrombin. When \( P_1 \) was either methionine or leucine, SI values were higher than 60 with elastase and lower than 3 with chymotrypsin (regardless of the \( P_2 \) residue). When the loop of the ACT/\( \alpha \)AT chimeras was shorter, SI values were higher for chymotrypsin, trypsin, and thrombin inhibition; the opposite was true for elastase. In contrast, the length of loop did not change the SI value with the ACT/PN1 chimeras. Thus SI value depended upon the sequence and the length of the reactive-site loop, as well as upon the target considered.

Attempts to detect SDS-stable complex formation followed incubation of a slight excess of serpin with \( \mu \)M quantities of the potential target (i.e., in conditions where cleavage reaction occurs substantially). Three patterns were observed (Fig. 1) as follows: 1) the formation of SDS-stable complex, but the presence of intermediate size fragments attributable to their degradation by remaining (active) protease (38); 2) absence of detectable complex, but accumulation of material migrating as ACT having the reactive-site loop cleaved; and 3) absence of detectable reaction (neither complex formation nor cleavage). Chimeras with leucine or methionine in \( P_1 \) position all formed an SDS-stable complex with chymotrypsin (pattern 1), whereas consistent with the high SI value, chimeras were mainly cleaved following incubation with elastase (pattern 2). Chimeras having arginine in \( P_1 \) position exhibited pattern 1 with thrombin, whereas the pattern was either 1 or 2 following incubation with trypsin (again, consistent with the high SI value, pattern was 2 with proline in \( P_2 \) and pattern 1 was otherwise). Regardless of the chimera, there was no clear evidence of SDS-stable complex formation nor of cleavage reaction with factor Xa, activated protein C, and urokinase; the predominant bands were those of the intact proteins (pattern 3). Finally, ACT/ATIII exhibited a mixed pattern. Thus, with the exception of trypsin, the pattern was specific for the target rather than for the chimera.

Under the conditions of the slow binding assays (low enzyme concentration and very large excess of serpin), depletion of the inhibitor by the cleavage reaction becomes negligible, as the total amount of cleaved inhibitor depends on the absolute amount of enzyme (30, 34–35). The \( k_{on} \) values obtained (Table II) did not reveal a clear relationship between the nature of the reactive-site loop and the specificity or function of the chimera, but in two instances only, the \( k_{on} \) values were higher when length of the reactive-site loop was shorter than that of ACT.

**Reactive-Site Loop of Antithrombin III**—Substitution of the reactive-site loop of ACT with that of antithrombin III transposed quite effectively the specificity of antithrombin III to ACT (Table II). ACT/ATIII exhibited \( k_{on} \) values within 10-fold those of antithrombin III for trypsin, thrombin, and factor Xa inhibition, whereas \( k_{on} \) values were lower than 10 M\(^{-1}\)s\(^{-1}\) with activated protein C and urokinase. Thus, ACT/ATIII exhibited a selectivity resembling that of antithrombin III. However, this apparent success must be tempered by the observation that simple substitution of the \( P_1 \) leucine with arginine largely accounts for the inhibitory properties of ACT/ATIII with trypsin or thrombin, whereas further substitution of the \( P_2 \) leucine with glycine is sufficient to mimic antithrombin III with every protease (30). Thus, substitution of the whole reactive-site loop did not alter the selectivity of the ACT mutant having the \( P_1 \) and \( P_2 \) residues of antithrombin III.

**Reactive-Site Loop of Protease Nexin 1**—Except for factor Xa inhibition, introducing the reactive-site loop of protease nexin 1 into ACT did not result in an appropriate transfer of specificity (Table II). Failure was most evident with urokinase; \( k_{on} \) values were at least 4 orders of magnitude lower than with protease nexin 1. In fact, regardless of the reactive-site loop, none of the ACT variants neutralized urokinase. The lack of urokinase inhibition was due to an absence of reaction rather than to the ACT variants acting as substrates; no cleavage reaction was detected by polyacrylamide gel electrophoresis. The \( k_{on} \) values for thrombin inhibition were also dramatically lower than with the loop donor (875- and 5000-fold with ACT/PN1 and ACT/PN1\(_{\text{des-TIVR}}\), respectively), and activated protein C inhibition was hardly detectable. However, the reactive-site loop of protease nexin 1 was functional in the context of the ACT framework. Less than 5-fold separated the \( k_{on} \) values of factor Xa inhibition by protease nexin 1 and ACT/PN1 and less than 27-fold separated those for trypsin inhibition. Factor Xa was also a remarkable exception, because shortening the loop increased the inhibitory activity of the chimera. Nevertheless, as was observed with the antithrombin III reactive-site loop, the effectiveness of transfer could be attributed to a predominant role of the \( P_1 \) and \( P_2 \) residues. Replacement of the \( P_2 \) leucine of ACT/\( \{P_1\text{-Arg}\} \) with alanine essentially mimics the behavior of ACT/PN1 (30).

**Reactive-site Loop of \( a_1 \)-Antitrypsin—\( a_1 \)-Antitrypsin with a methionine in \( P_1 \) position inhibits elastase much more efficiently than ACT (\( k_{on} \) value 10-fold higher) and chymotrypsin 14-fold more efficiently (36, 37). Yet the \( k_{on} \) values obtained with the ACT/\( \alpha \)AT chimera were 2- to 3-fold lower than those with ACT, and truncation of the loop further decreased the \( k_{on} \) values 2- to 4-fold (Table III). Thus, grafting the \( a_1 \)-antitrypsin reactive-site loop into ACT not only failed to reproduce the specificity of \( a_1 \)-antitrypsin, but it was rather detrimental for the inhibitory activity of ACT toward chymotrypsin and elastase.

Behavior of the ACT chimera carrying the loop of \( a_1 \)-antitrypsin (\( P_1\text{-Arg} \)) was somewhat erratic (Table II). Single replacement for proline of the \( P_2 \) leucine of ACT/\( \{P_1\text{-Arg}\} \) does not change the \( k_{on} \) value for trypsin inhibition (30); replacement of the entire loop increased this value 6-fold. In contrast, the \( P_2 \) mutation improved the \( k_{on} \) values over 18-fold for thrombin and factor Xa inhibition (30), whereas substitution of the entire loop

![Fig. 1. Trypsin interaction with the various ACT chimeras. Analysis by SDS-polyacrylamide gel electrophoresis (gradient 10–20% acrylamide) of the reaction of trypsin (1 \( \mu \)M) with the various ACT chimeras prepared (2 \( \mu \)M). Lane 1, molecular mass standards; lane 2, recombinant ACT; lane 3, trypsin; lanes 4–9 trypsin incubated for 30 min at room temperature with ACT/\( \{P_1\text{-Arg}\} \), ACT/\( \alpha \)AT/\( \{P_1\text{-Arg}\} \), ACT/PN1, ACT/\( \alpha \)AT/PN1, ACT/PN1\(_{\text{des-TIVR}}\), ACT/ATIII, respectively. Lane 10, recombinant ACT cleaved within the reactive-site loop by the venom from Bitis arietans. In patterns 1, formation of an SDS-stable complex occurs, but partial degradation results in formation of intermediate size products; in pattern 2 the major product is the chimera having its reactive-site loop cleaved.](image)
Each slow-binding inhibition experiment was performed at least twice; the \( k_{on} \) value given represents the weighted mean of these determinations. The standard errors of the weighted means were 5% or less of the mean value. Abbreviations used are: FXa, factor Xa; APC, activated protein C; ND, not determined. Values of the ACT double mutants (P1 arginine and either P2 proline, alanine or glycine) were taken from Djie et al. (30).

### Table II

|                     | Trypsin | Thrombin | FXa | APC | Urokinase |
|---------------------|---------|----------|-----|-----|-----------|
| \( k_{on} \) \( (M^{-1} s^{-1}) \) |          |          |     |     |           |
| ACT(P1=Arg)         | \( 4.1 \times 10^5 \) | \( 1.8 \times 10^3 \) | \( 2.1 \times 10^1 \) | \( 1.9 \times 10^2 \) | <10         |
| Antithrombin III    | \( 1.4 \times 10^5 \) | \( 1.1 \times 10^4 \) | \( 3.1 \times 10^3 \) | <10        | <10        |
| ACT/ATIII           | \( 3.7 \times 10^5 \) | \( 1.4 \times 10^4 \) | \( 7.0 \times 10^3 \) | <10        | <10        |
| ACT(P1=Arg; P3=Gly) | \( 5.5 \times 10^5 \) | \( 1.5 \times 10^4 \) | \( 3.0 \times 10^3 \) | <10        | ND         |
| Protease nexin 1    | \( 4.7 \times 10^5 \) | \( 2.1 \times 10^4 \) | \( 6.5 \times 10^3 \) | \( 5.2 \times 10^3 \) | \( 2.9 \times 10^5 \) |
| ACT/PNI             | \( 1.8 \times 10^5 \) | \( 2.4 \times 10^3 \) | \( 1.4 \times 10^3 \) | \( 1.4 \times 10^3 \) | <10        |
| ACT/PNI \(_{des-TIVR}\) | \( 2.7 \times 10^4 \) | \( 4.2 \times 10^2 \) | \( 2.5 \times 10^3 \) | <10        | <10        |
| ACT(P1=Arg; P3=Ala) | \( 4.2 \times 10^5 \) | \( 6.3 \times 10^4 \) | \( 6.0 \times 10^4 \) | \( 7.8 \times 10^4 \) | ND         |
| \( \alpha \)-Antitrypsin/P1=Arg) | \( 4.2 \times 10^5 \) | \( 3.1 \times 10^4 \) | \( 1.9 \times 10^4 \) | \( 7.0 \times 10^4 \) | \( 8.9 \times 10^4 \) |
| ACT/AT(P1=Arg)      | \( 2.6 \times 10^5 \) | \( 4.9 \times 10^3 \) | \( 9.0 \times 10^3 \) | \( 4.8 \times 10^4 \) | <10        |
| ACT/AT(P1=Arg; des-TIVR) | \( 3.2 \times 10^4 \) | \( 8.6 \times 10^2 \) | \( 2.0 \times 10^3 \) | <10        | <10        |
| ACT(P1=Arg; P3=Pro) | \( 4.4 \times 10^5 \) | \( 3.3 \times 10^4 \) | \( 6.8 \times 10^3 \) | \( 2.0 \times 10^4 \) | ND         |

### Table III

\( k_{on} \) values for chymotrypsin and elastase of the chimeras having a P5 leucine or methionine

\( k_{on} \) values were determined as in Table II. The \( k_{on} \) values for inhibition by \( \alpha \)-antitrypsin values were taken from Beatty et al. (36) or Hopkins et al. (43).

|                     | Chymotrypsin \( k_{on} \) \( (M^{-1} s^{-1}) \) | Elastase \( k_{on} \) \( (M^{-1} s^{-1}) \) |
|---------------------|----------------------------------|-------------------------------------|
| ACT                 | \( 4.1 \times 10^5 \)               | \( 1.0 \times 10^4 \)               |
| ACT \(_{des-TIVR}\) | \( 9.0 \times 10^4 \)               | \( 1.3 \times 10^5 \)               |
| \( \alpha \)-antitrypsin | \( 5.9 \times 10^5 \)           | \( 1.2 \times 10^7 \)           |
| ACT/AT              | \( 1.3 \times 10^5 \)               | \( 6.2 \times 10^3 \)               |
| ACT/AT(P1=Arg)      | \( 3.1 \times 10^4 \)               | \( 2.7 \times 10^3 \)               |

improved marginally (less than 5-fold) the \( k_{on} \) values. Behavior with activated protein C was again different; replacement of the P5 residue is neutral (30), but substitution of the entire loop was detrimental (4-fold decreased of the \( k_{on} \) value). Thus, in contrast to the antithrombin III and protease nexin 1 loop transfers, replacement of the P2 residue did not mimic the effect of swapping the entire loop with \( \alpha \)-antitrypsin. In particular, three of the chimeras were less effective than the ACT mutant having the same P1 and P2 residues, suggesting that one or more detrimental elements outweighed the benefit of the P1 and P2 substitutions. The length of the loop was particularly critical for trypsin inhibition: ACT/AT(P1=Arg; des-TIVR) inhibited trypsin with a \( k_{on} \) value 80-fold lower than that of ACT/AT(P1=Arg), whereas the \( k_{on} \) values decreased 6-fold at the most with thrombin and factor Xa.

**DISCUSSION**

The reactive-site loop of serpins undoubtedly defines in part their ability to inhibit a particular protease (6, 30, 39–44). Therefore, we anticipated that exchanging loops between serpins might reassign the targets. Although swapping of reactive-site loops dramatically modified the specificity, in general it was not possible to transfer the inhibitory properties of a loop donor to ACT. Overall, the specificity of the chimera rarely matched that of the loop donor, indicating that other players must participate (directly or indirectly) in the specificity of serpins. In addition to residues P4 to P9, at least three factors could conceivably influence the serpin behavior: (i) length of the reactive-site loop, (ii) secondary binding sites remote from the reactive-site loop, and (iii) intramolecular control of the reactive-site loop conformation. Failure to transfer a given specificity to ACT would result from an impaired mechanism of action due to one or more of these factors.

**Length of the Reactive-site Loop**—There was no clear relationship between length of the reactive-site loop and specificity, but as a general rule, ACT chimera exhibited higher \( k_{on} \) value with the longer loop. Thus our data favor the hypothesis that the length of the loop is important for the mechanism of inhibition within a given framework, rather than in governing the specificity per se. This is consistent with the observation that specificity does not correlate with length of the reactive-site loop in natural serpins. ACT and \( \alpha \)-antitrypsin both inhibit chymotrypsin, even though the length of their loops differs by 4 amino acids. Similarly, the loops of \( \alpha \)-antitrypsin and antithrombin III differ by 3 amino acids, but both inhibit trypsin. Also consistent with the length of the loop being important for the serpin mechanism of action rather than its specificity, Avron et al. (45) have shown that insertion of an alanine in P5 position decreases the \( k_{on} \) value of \( \alpha \)-antitrypsin for elastase 10-fold and for trypsin nearly 100-fold. However, two exceptions prohibit generalization and suggest that deletion of 4 amino acids from the reactive-site loop of ACT did not hamper its folding nor its mechanism of action. Elastase was inactivated more rapidly by ACT/AT(P1=Arg) than by ACT, and compared with ACT/PNI, factor Xa was inactivated more rapidly by ACT/PNI \(_{des-TIVR}\). Thus, the length of the reactive-site loop appears important in the context of the serpin framework but alone does not account for the overall failure in transposing specificity.

**Secondary Binding Sites**—Several serpins possess a secondary binding site, often remote from the reactive-site loop, which promotes interaction with the target. The binding site interacts with a complementary motif on the protease, often distant from the catalytic groove. The specificity would be controlled by the simultaneous presence of these motifs on the inhibitor and its target. Swapping frameworks would shuffle the secondary motifs, rendering the serpins' behavior relatively unpredictable. For example, plasminogen activator inhibitor 1 uses its acidic residues P4’, P5’, and P9 to interact with a positively charged surface-loop (amino acids 35 to 41)² of tissue plasminogen activator (48–50), and heparin cofactor II uses its negatively charged N-terminal extension to interact with anion-binding exosite 1 of thrombin (46, 47). Likewise, antithrombin III uses an unknown motif to interact with the 60-loop insertion of thrombin (44), and protease nexin 1 uses an unknown motif to interact with urokinase (51). Precisely, regardless of the reactive-site loop sequence, none of our ACT variants inhibited urokinase. Thus, our results would be consistent with the hypoth-

³The chymotrypsin numbering system, based on topological identities, is used for all serine protease residues mentioned in this study.
Intramolecular Control of Serpin’s Specificity

that for urokinase inhibition a critical area of protease nexin 1 had not been implanted in the ACT framework. Alternatively, a motif in ACT could interact favorably with elastase, chymotrypsin, and perhaps factor Xa, while being detrimental for activated protein C and urokinase binding. In this regard, it is interesting to note that ACT binds double-stranded DNA, through a stretch of 3 lysines (residues 212–214), a property not shared by α1-antitrypsin, antithrombin III, or protease nexin 1 (15, 52). From x-ray structure data, this DNA binding site must be close to the 35–41 surface loop of the target protease, at least during formation of the initial complex. The 35–41 surface loop of factor Xa is negatively charged, whereas the same P3–P3

Intramolecular Control—In several instances, substitution of the entire loop was more detrimental than simple substitution of the corresponding P1 and P2 residues. This observation suggests that the specificity is also restrained by intramolecular interactions. Residues within the core of the inhibitor would control the specificity by ensuring proper folding and/or function of the reactive-site loop. Important intramolecular interactions between the reactive-site loop and the core of the molecule have been characterized in several canonical inhibitors (3, 53). In Kunitz-type 1 inhibitors, for instance, the conformation of the reactive-site loop is firmly maintained by a disulfide bond between Cys14 (P1 position) and Cys38 (51) in the core of the inhibitor (54). This covalent link allows the reactive-site loop of Kunitz inhibitors to withstand extensive substitution in most positions (other than P3), with the mutant specificity following the preferences of the protease target (55, 56). In eglin c, a hydrogen bonding network stabilizes the conformation of the P1–P4 bond (57); disruption of this network allows greater mobility of the reactive-site loop, resulting in eglin c becoming a substrate of elastase (58). Consistent with the hypothesis that serpin specificity relies on intramolecular control, the reactive-site loops of ACT and α1-antitrypsin are both suitable for chymotrypsin inactivation but are more efficient in the context of their original framework. The crystal structure of the ACT/αAT chimera reveals that only a few contacts occur between the reactive-site loop and the underlying protein (16), whereas the same P8–P9 sequence in the context of α1-antitrypsin has developed many more contacts and packs close to the main body of the serpin (59–60). The loose reactive-site loop of ACT/αAT inactivates chymotrypsin 55-fold less efficiently than ACT (28); the same loop tightly packed in α1-antitrypsin is 328 times more effective.

Thus, loss of an intramolecular control would explain that several specificity swaps failed, even though donor and acceptor have a common target. Yet, at least in one instance loop swapping seems feasible without constraint, because this is the natural process to generate multiple serpins from a single gene in Manduca sexta (61–62). Using a unique N-terminal framework, the insect generates 12 serpins by mutually exclusive exon shuffling. Among the serpins produced, the reactive-site loop varies in sequence as well as in length (by as many as 6 residues). Perhaps a key difference between the chimeras described in this paper and those of M. sexta resides in the C-terminal portion of the molecule. Both the reactive-site loop and the C-terminal portions are systematically exchanged in the insect serpins, whereas in our ACT chimeras, the C-terminal portion remained that of ACT. It is possible that the C-terminal region (strands 4 and 5 of the B β-sheet) plays an unidentified role in serpin function.
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