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Evolution of Serpin Specificity: Cooperative Interactions in the Reactive-Site Loop Sequence of Antithrombin Specifically Restrict the Inhibition of Activated Protein C

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Abstract. Protease cascades and their inhibitors are a common feature of many biological regulatory systems, and the various components of such cascades have been subjected to a long and concerted evolution. We present here evidence that in the coagulation cascade, the sequence of the protease-binding reactive-site loop of antithrombin has evolved such that the majority of its residues has been acquired not for the efficient inhibition of its target proteases, thrombin and factor Xa, but to avoid the inhibition of activated protein C (APC). We substituted residues of the reactive-site loop of antithrombin into α_1-antitrypsin and tested the chimeras against thrombin, factor Xa, and APC. With respect to factor Xa and thrombin, the difference in association rate between the fastest and the slowest inhibitors was 5.5- and 88-fold, respectively. However, with respect to APC the difference was 12,500-fold. While most of the variation in the inhibition rates of thrombin could be accounted for by P2 Gly-to-Pro substitutions, for APC almost every residue had an effect on inhibition. In 22 of 25 direct comparisons of antitrypsin residues with antithrombin residues, either singly or in blocs, the antithrombin residues caused a decrease in the rate of inhibition of APC. The antithrombin residue Asn393, at position P′3, emerged as particularly important for avoiding the inhibition of APC, however, its 190-fold effect was seen only when in conjunction with antithrombin P7 to P′2 residues. Cooperative effects among residues of the reactive-site loop thus emerged as critical for restricting the activity of this sequence against APC.

Key words: Serpin — Antithrombin — α_1-Antitrypsin — Activated protein C — Thrombin — Factor Xa — Kinetics — Cooperativity

Introduction

The serpin family of serine protease inhibitors play a critical role in many biochemical cascades, including apoptosis, hemostasis, and complement activation (Potempa et al. 1994). Thrombin is the pivotal protease of the coagulation cascade (reviewed by Davie et al. 1991; Furie and Furie 1988) and is generated from its zymogen, prothrombin, by the action of the prothrombinase complex, an assembly of factors Xa and Va on phospholipid surfaces. The activity of thrombin is regulated in many ways; among these are direct inhibition by the serpin antithrombin (which also inhibits factor Xa) and a thrombin/thrombomodulin-activated feedback mechanism involving activated protein C (APC). In combination with the cofactor protein S, APC suppresses the further gen-

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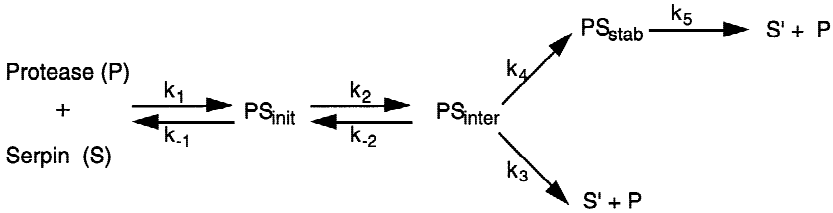


Scheme I. General serpin reaction mechanism. P, protease; S, unreacted serpin; S_{init}, the serpin in the initial encounter complex; S_{inter}, the serpin in an intermediate complex; S′, the free cleaved serpin; S_{stab}, the serpin when in a stable complex with the protease. After initial binding of a serpin to a protease, one of two reaction paths may be taken, one which leads to the formation of a tight complex between the protease and the inhibitor and one to the release of cleaved, inactive inhibitor and active enzyme (Olson et al. 1995; Patston et al. 1991; Stone and Le Bonniec 1997). In addition to the possible generation of cleaved, inactive inhibitor during the inhibition process, serpin–enzyme complexes eventually dissociate to yield inactive inhibitor and active enzyme (the step governed by k_5), though usually on time scales too long to be biologically significant.

eration of thrombin by inactivating the prothrombinase complex. Both antithrombin and activated protein C have separately been shown to be critical for the *in vivo* regulation of thrombosis (Esmon 1989; Perry 1994).

A typical serpin is an approximately 400-amino acid protein which operates as a "mechanism-based" inhibitor (see Scheme I). Central to the nature of mechanism-based inhibition is the catalytic attack of the protease on the inhibitor, which occurs at the P1–P′1 bond in the reactive-site loop (RSL),[1] an exposed flexible region some 20 amino acids in length (see Fig. 1). It is thought to be the persistence of an acyl bond between the carbonyl carbon of the P1 residue of the serpin and the γO of the catalytic serine which results in pseudo-irreversible inhibition (Lawrence et al. 1995; Olson et al. 1995; Stone and Le Bonniec 1997; Wilczynska et al. 1995). Given that serpins exploit the natural catalytic activity of the protease for its inhibition, it is reasonable to assume that the RSL sequence of cognate serpins will resemble that of substrates for the cognate protease. Indeed, among most inhibitory serpins, the nature of the P1 residue is found to be tightly correlated with the target preferences of the cognate protease.

The three proteases, thrombin, factor Xa, and APC, all have a preference for Arg at P1 and, *a priori*, might all be expected to be reasonably well inhibited by antithrombin, which also has an Arg at P1. However, with respect to these three proteases, antithrombin is extremely specific. This is caused mostly by its remarkably low second-order association rate constant (k_{ass}) for APC [0.014×10^1 M^{-1} s^{-1} (Hermans and Stone 1993)]; the difference in the k_{ass} values for its association with thrombin [k_{ass} 1.1×10^4 M^{-1} s^{-1} (Wallace et al. 1989)] versus APC is thus almost 10^5-fold, and that between factor Xa [k_{ass} 2.3×10^3 M^{-1} s^{-1} (Olson et al. 1992)] and APC is over 10^4-fold. The reaction of antithrombin with both thrombin and factor Xa is greatly accelerated by the cofactor heparin (Nesheim 1983; Olson et al. 1992). Heparin accelerates the reaction with thrombin via the template mechanism, where the binding of a thrombin molecule and an antithrombin molecule to the same strand of heparin results in their more rapid approximation and subsequent reaction (Olson and Björk 1992; Olson and Shore 1986) and, thus, does not appear directly to involve the RSL. In contrast, the mode of the acceleration of the reaction with factor Xa is dependent on the structure of the RSL, altering its structure such that it reacts more readily with factor Xa (Chuang et al. 1999; Pike et al. 1997). While high concentrations of heparin also increase the inhibition of APC by antithrombin, it remains modest, at below 8.0×10^1 M^{-1} s^{-1} (Hermans and Stone 1993; Rezaie 1996), thus retaining the large difference in inhibition rates between thrombin and factor Xa, and APC. The molecular basis for this specificity has remained elusive.

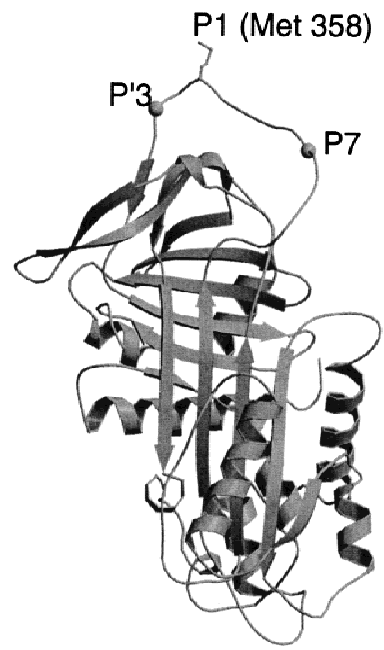


Fig. 1. Line drawing of wild-type α_1-AT derived from the coordinates of Elliott et al. (1996). The P1 residue (met358) side chain is fully drawn, and the Cα of RSL residues P7 (Phe352) and P′3 (Pro361) are shown as black dots.

α_1-Antitrypsin (α_1-AT) is colocalized with these three proteases in plasma at a high concentration (~50 μ*M*) but

[1] The nomenclature for residues of the reactive-site loop of serpins is based on that of Schechter and Berger (1967). In α_1-AT the P1 residue is Met358.

is a poor inhibitor of all three, for two reasons: peptide loops surrounding the active sites of thrombin and APC contain residues, in particular, E192, restricting their inhibition by α_1-AT (Le Bonniec and Esmon 1991; Rezaie and Esmon 1993), and the presence of a methionine at P1 in α_1-AT reduces the association rate with all three. Mutation of the P1 residue from Met to Arg increases the rate of inhibition of all three proteases by α_1-AT by up to 10^5-fold (Lewis et al. 1978; Travis et al. 1986). However, if the antithrombin residues from P7 to P′3 are substituted into the α_1-AT RSL, most of antithrombin's specificity is conferred on the chimera (Hopkins et al. 1995). Contrary to the poor reaction rate of α_1-AT, or the moderately fast rate of the P1-Arg α_1-AT mutant, with all these proteases, this chimera inhibited thrombin and factor Xa at rates comparable to that of unheparinized antithrombin and also had an extremely low association rate with APC. Furthermore, few of the residues of antithrombin's RSL are optimal for binding to its primary target, thrombin. Thrombin's specificity has been extensively mapped (Backes et al. 2000; Le Bonniec et al. 1996; Lottenberg et al. 1983; Vindigni et al. 1997), and only the P1 and P′1 residues of antithrombin's RSL correspond to optimal sequences for binding to thrombin's active-site cleft. These data therefore suggested that the evolution of antithrombin's RSL sequence has in fact been driven not only by the requirements for the inhibition of thrombin and factor Xa, but also to avoid the concurrent inhibition of activated protein C.

To support better the concept of such "negative selection," we have made sequential substitutions of portions of antithrombin's RSL into the α_1-AT RSL and determined the activity of the chimeras against thrombin, factor Xa, and APC. Grafting the residues into α_1-AT excluded possible contributions of antithrombin regions external to the RSL from the outset. Given the poor association rate of α_1-AT with all three enzymes, and of the P7- to P′3-substituted chimera with APC, it was also safe to assume that regions external to the α_1-AT RSL were unlikely to have much direct impact on inhibition rates.

Materials and Methods

Materials. The substrates S-2266 and S-2222 were purchased from Kabi-Pharmacia (Mölndal, Sweden). Bovine serum albumin and *p*-nitrophenyl *p′*-guanidinobenzoate were purchased from Sigma (Poole, UK). Thrombin was prepared as described previously (Stone and Hofsteenge 1986). APC was a gift from Drs. J. Stenflo and A. Öhlin (Malmö, Sweden). Factor Xa was obtained from Boehringer Mannheim. α_1-AT mutants were produced in *Escherichia coli* as described previously (Hopkins and Stone 1995).

Determination of Kinetic Parameters. All assays were performed at 37°C in 30 m*M* sodium phosphate, pH 7.4, containing 0.16 *M* NaCl, 0.1% polyethylene glycol (M_r 4000), 0.2 mg/ml protease-free bovine serum albumin, and 200–400 μ*M* S-2266 or S-2222. The proteases were titrated with *p*-nitrophenyl *p′*-guanidinobenzoate, and serpins were titrated as described previously (Djie et al. 1997; Hopkins et al. 1995). The serpin concentration was also estimated by Bradford assay; recombinant α_1-AT served as a standard. The stoichiometry of inhibition was estimated by dividing the concentration obtained from Bradford assay by that obtained by titration. Monitoring of slow binding inhibition was performed using a Hewlett Packard diode-array spectrophotometer by measuring the production of *p*-nitroanilide between 400 and 410 nm. For the analysis of APC inhibition the plastic cuvettes were coated by incubating with 10 mg/ml bovine serum albumin and 0.1% polyethylene glycol (M_r 8000) overnight. The cuvettes were subsequently washed in distilled water and dried at 37°C before use. Each progress curve experiment consisted of six or seven assays, one in the absence of serpin and the others with increasing serpin concentrations. After initiation of the reactions by the addition of either thrombin, factor Xa, or APC, the release of *p*-nitroanilide was monitored. Data points where substrate utilization was in excess of 10% of the total substrate concentration were excluded from the analyses. The data were fitted to an equation describing slow tight-binding inhibition corrected for the competition introduced by the substrate by the factor $1 + S/K_m$. The K_m values for S-2266 with thrombin and APC were 262 ± 14 and 654 ± 62 μ*M*, respectively (Hermans and Stone 1993). The K_m value of S-2222 with thrombin was 103 ± 10 μ*M*, and that with factor Xa was 547 ± 25 μ*M*. In cases where the reaction rate was too slow to be estimated in the above manner, it was performed under second-order conditions as follows (Beatty et al. 1980). The serpin was incubated in a microtiter plate in the above buffer at 37°C; at timed intervals the enzyme was added to individual wells to an equimolar final concentration. After 4 to 10 h, the reactions were diluted with buffer containing chromogenic substrate and the residual protease activity was estimated from the substrate conversion rate over a period of 10 min. Data points where substrate consumption exceeded 10% of the initial concentration were not used in the subsequent calculations.

Results

Effect of Substituting α_1-AT Residues for Antithrombin Residues on the Reaction Kinetics with Thrombin, Factor Xa, and APC

To investigate the role of different residues of antithrombin's RSL in its specificity, various portions of this loop were substituted into the α_1-AT RSL. Starting from our previously reported chimera, LS7-3′[2] (indicated also by the letter "j" in Table 1), we truncated the substituted region on both the P side and the P′ side to define the minimal sequence of the antithrombin RSL which would confer a high degree of discrimination among the inhibition of thrombin, factor Xa, and APC on the chimera. Table 1 shows the second-order association rate constant (k_{ass}) for the inhibition of thrombin, factor Xa, and APC by the chimeras. Table 1 shows a clear distinction among the effects of many substitutions on either thrombin, factor Xa, or APC. Excluding the P1 Met → Arg substitution, the difference in k_{ass} between the best and the worst

[2] In the chimerical mutants of α_1-AT, α_1-AT residues replaced by antithrombin residues are defined, e.g., LS7–3′ is an α_1-AT-antithrombin "loop swap" with α_1-AT having the P7 to P′3 residues of antithrombin instead of its own.

Table 1. Second-order association rate constants of natural and mutant serpins with factor Xa, thrombin, and APC*

		Reactive-site loop sequence												k_{ass} (M^{-1} s^{-1})		
Serpin		P8							P1				P′4	Thrombin	APC	Factor Xa
α_1-AT		M	F	L	E	A	I	P	M	S	I	P	P	4.8×10^1†	1.1×10^1‡	nd
ATIII		T	A	V	V	I	A	G	R	S	L	N	P	1.10×10^4§	0.014×10^1‖	1.80×10^3
LS1	a	–	–	–	–	–	–	–	R	–	–	–	–	4.80×10^5#	7.00×10^4#	4.10×10^4
LS1/2′	b	–	–	–	–	–	–	–	R	–	L	–	–	1.80×10^5	2.60×10^4	3.10×10^4
LS1/3′	c	–	–	–	–	–	–	–	R	–	–	N	–	6.40×10^5	1.60×10^4	1.50×10^4
LS1–3′	d	–	–	–	–	–	–	–	R	–	L	N	–	5.99×10^5	9.77×10^3	1.90×10^4
LS2–3′	e	–	–	–	–	–	–	G	R	–	L	N	–	6.72×10^4	6.60×10^2	nd
LS3–3′	f	–	–	–	–	–	A	G	R	–	L	N	–	1.55×10^4	1.18×10^2	2.50×10^4
LS4–3′	g	–	–	–	–	I	A	G	R	–	L	N	–	2.41×10^4	4.2×10^1	7.5×10^4
LS5–3′	h	–	–	–	V	I	A	G	R	–	L	N	–	4.44×10^4	2.0×10^1	5.50×10^4
LS6–3′	i	–	–	V	V	I	A	G	R	–	L	N	–	4.13×10^4	1.8×10^1	2.40×10^4
LS7–3′	j	–	A	V	V	I	A	G	R	–	L	N	–	4.60×10^4#	0.56×10^1#	3.76×10^4
LS7–2′	k	–	A	V	V	I	A	G	R	–	L	–	–	4.80×10^4	1.10×10^3	1.30×10^4
LS7–1	l	–	A	V	V	I	A	G	R	–	–	–	–	3.22×10^4	3.48×10^2	2.00×10^4
LS7–3/P1R	m	–	A	V	V	I	A	–	R	–	–	–	–	1.37×10^6	2.40×10^3	1.70×10^4
LS2–1	n	–	–	–	–	–	–	G	R	–	–	–	–	1.30×10^4#	4.30×10^3#	1.60×10^4
LS7–3′/P2P	o	–	A	V	V	I	A	–	R	–	L	N	–	3.10×10^5#	4.90×10^2#	1.60×10^4
LS7–3/P′2L	p	–	A	V	V	I	A	–	R	–	L	–	–	1.34×10^6	4.16×10^2	1.60×10^4

* Each value was determined at least twice, and the mean of the estimations is presented; all estimates of k_{ass} had standard errors of less than 10%. ATIII, antithrombin. In the chimeras, residues identical to α_1-AT are represented by a dash. Lowercase letters to the left of the sequences are used in Fig. 2 to define the serpins compared therein

† Travis et al. (1986)

‡ Heeb et al. (1990)

§ Wallace et al. (1989)

‖ Hermans and Stone (1993)

Hopkins et al. (1995)

inhibitors of factor Xa is approximately 5.5-fold (LS4-3′ and LS7-2′; g and k). The best and worst inhibitors of thrombin differed by 88-fold (LS7-3/P1R and LS3-3′; m and f); most of this difference could be accounted for by P2 Pro → Gly substitutions. However, the range of the effect of the mutations on the inhibition of APC is substantially higher, at 12,500-fold, the best being LS1 (a) at 7×10^4 M^{-1} s^{-1} and the worst being LS7-3′ (j) at 0.56×10^1 M^{-1} s^{-1}.

The loss of activity against APC could be seen to be progressive; in general, the greater the number of antithrombin residues present in the chimera, the lower the k_{ass} with APC. The P1, P2, and P′3 residues had the largest individual effects. Nevertheless, while none of the P3 to P7 residues, individually or collectively, had a large effect on the inhibition of thrombin or factor Xa, their cumulative effect on the inhibition of APC reached 116-fold (compare LS2-3′ and LS7-3′; e and j).

No significant effect was observed on the stoichiometry of inhibition for any of the variants examined, with any of the proteases, all being less than 2 (data not shown).

Cooperativity in the Determination of Specificity

The P2 Pro → Gly mutation had one of the more significant effects on the rate of inhibition of both thrombin and APC by the chimeras. However, the effect of a P2 Pro → Gly substitution on the rate of inhibition of APC varied from a 2- to 3-fold increase in k_{ass} (LS7-3/P′2L to LS7-2′; p → k) to an 87-fold decrease (LS7-3′/P2P to LS7-3′; o → j), indicating a strong context-dependent effect of this mutation. Further context-dependent effects were sought by arranging the data in Table 1 so as to illustrate the effects of identical mutations in different contexts; the results are shown in Fig. 2. Figure 2 shows that for 22 of 25 direct comparisons of α_1-AT for an antithrombin residue(s) in different contexts, singly or in blocs, the antithrombin residue(s) cause a decrease in the association rate with APC. Figure 2 also shows that context-dependent effects on the inhibition of APC are not restricted to the P2 residue: for all residues, or blocs of residues, where an identical mutation was present in two or more chimeras, a context-dependence of effect can be found. The largest such effect is seen with the P′3 Pro → Asn mutation, which had a 196-fold effect on the inhibition rate of APC in the transition from LS7-2′ to LS7-3′ (k → j), a 4.3-fold effect when only this residue is mutated (LS1 to LS1/3′; a → c), and no significant effect in the transition from LS7-3/P′2L to LS7-3′/P2P (p → o). Analysis of these mutations by constructing double mutant cycles according to Horovitz (1987) reveals a number of cooperative effects, the largest being a 45-fold effect between the P′3 Asn and the P7 to P′2 residues (Scheme II). No individual residues responsible for cooperating with the P′3 Asn were identified. How-

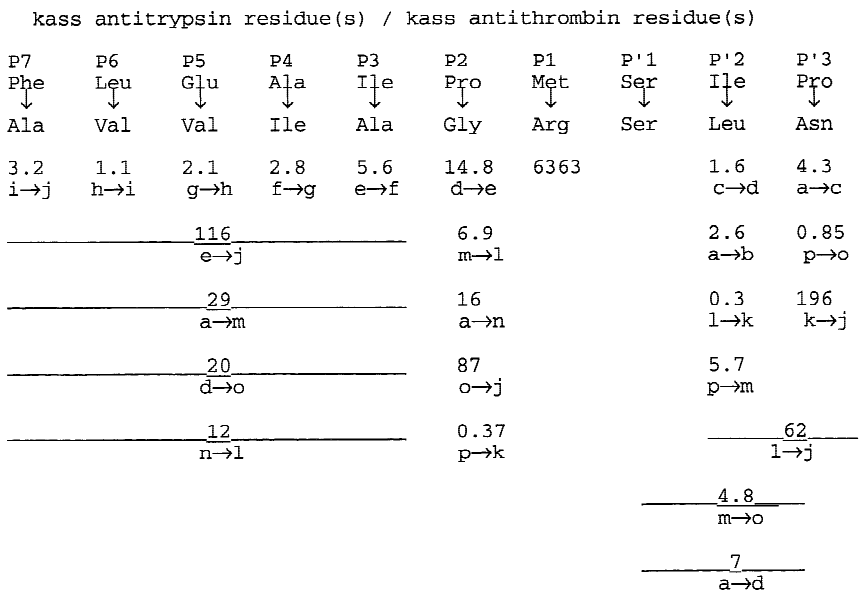
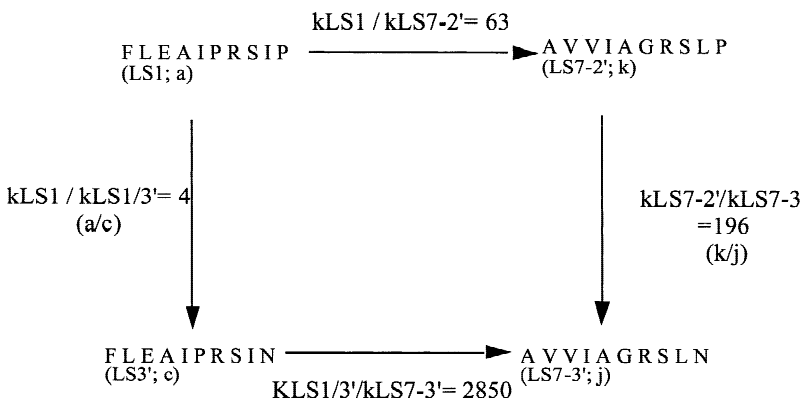


Fig. 2. Context dependence of the effect of antithrombin residues substituted into the reactive-site loop of α_1-AT. The numbers refer to the ratio of the k_{ass} values against APC for variants with α_1-AT residues in indicated positions to those for the antithrombin residues in the same positions. Lowercase letters indicate which variants from Table 1 are being compared. Horizontal bars indicate that more than one residue has been substituted; the residues in question are those spanned by the bar.



Scheme II. Cooperative effects involving the P′3 Asn and the P7 to P′2 residues of antithrombin. The identical P′3 Pro-to-Asn mutation has an approximately 45-fold greater effect in the context of the antithrombin P7 to P′2 residues than in the context of the α_1-AT P7 to P′2 residues.

ever, a cycle similar to that in Scheme II where LS7-2′ (k) and LS7-3′ (j) are replaced by LS7-3/P′2L (p) and LS7-3′/P2P (o), respectively, showed that in the presence of a P2 Pro the cooperative effect between the P′3 Asn and the P7–P2 residue effect was reduced from 45- to 5-fold.

Smaller cooperative effects, ranging from two- to fourfold, were also observed which reduced the inhibition rate of thrombin. As with the inhibition of APC, these involved mostly the P′ region cooperating with the P-region: for example, an approximately fourfold cooperative effect exists between the P2Gly and the P′2Leu and P′3Asn, which reduces the association rate with thrombin [compare the 37-fold effect in the transition from LS1 (a) to LS2-1 (n) with the 9-fold effect in the transition from LS1-3′ (d) to LS2-3′ (e)].

Discussion

In addition to rapid inhibition of cognate proteases, inhibitor specificity implies a lack of inhibition of nontarget proteases. In this work we have provided evidence that the RSL sequence of antithrombin has evolved specifically to restrict the inhibition of APC. The residues of this sequence act in both cooperative and noncooperative fashions to achieve this effect.

Despite the *in vivo* significance of the inhibition of factor Xa by antithrombin, we found little effect of our mutations on its inhibition. It nevertheless remains likely that the substitutions we made were neutral with respect to the inhibition of factor Xa and that others would have shown a more dramatic effect, as demonstrated previously (Theunissen et al. 1993). The P2 Gly appears to play important roles in the inhibition of factor Xa by antithrombin, permitting structural modifications induced by specific pentasaccharide heparin and a consequent dramatic increase in the rate of inhibition of factor Xa by antithrombin (Chuang et al. 1999; Pike et al. 1997). Such an effect would obviously not have been observed here. Wild-type and P2Pro antithrombin may be compared with the analogous α_1-AT chimeras (LS7-3′ and LS7-3′/P2P; j and o). There is a notable difference between the inhibition of thrombin and that of factor Xa

when these various serpins are compared. The difference between the inhibition rates of thrombin by wild-type antithrombin and P2Pro antithrombin, with or without pentasaccharide, and the analogous chimeras shown here is small (between two- and fourfold), with the α_1-AT chimeras having a higher association rate. For the inhibition of factor Xa in the absence of pentasaccharide, the α_1-AT chimeras were faster inhibitors than wild-type antithrombin and P2Pro antithrombin, by 7- and 13-fold, respectively. However, in the presence of pentasaccharide the order was reversed, and the wild-type and P2Pro antithrombins became 20-fold better inhibitors of factor Xa than the analogous α_1-AT chimeras. This suggests a role for antithrombin RSL residues outside the P7 to P′3 region for the inhibition of factor Xa, but not for thrombin. The serpin RSL is conserved in sequence beyond P7, and therefore residues more N-terminal than this are unlikely to be direct specificity determinants. On the P′ side, residue P′4 has been shown to be an important determinant for the specificity of PAI-1 toward tPA (Madison et al. 1989, 1990); the P′5 Glu of PAI-1 has also been shown to be important for avoiding the inhibition of thrombin by PAI-1 (Rezaie 1998). While the P′4 Pro is identical in antithrombin and α_1-AT, the P′5 residue of α_1-AT is a Glu and that of antithrombin is an Asn. The P′5 Asn of antithrombin is therefore an interesting candidate for pentasaccharide-activated factor Xa-specific functions of the antithrombin RSL sequence.

With respect to the inhibition of thrombin, most of the variation in reaction rates among our chimeras could be attributed to the presence of a Gly or Pro at P2. Previous studies have indicated that thrombin has a marked preference for Pro at P2 (Backes et al. 2000; Lottenberg et al. 1983; Vindigni et al. 1997). In this work, the observed effect of a P2Gly → Pro mutation on the rate of thrombin inhibition varied from a 7- to a 42-fold increase. Interestingly, it was in the variants with the largest number of antithrombin residues that the P2Gly → Pro mutation had its lowest effect: when the P′2 and P′3 residues are as in antithrombin, the effect of the P2Gly → Pro mutation is diminished and becomes of a size similar to that observed when the same mutation is made in antithrombin itself (Chuang et al. 1999; Sheffield and Blajchman 1994). Our results therefore allowed us to deduce a cooperative effect between the P2 Gly and the P′2 and P′3 residues, which reduces the association rate with thrombin: in the presence of the α_1-AT P′2 and P′3 residues, the effect of the P2Gly → Pro mutation is fourfold larger than in the presence of the antithrombin P′2 and P′3 residues.

The most dramatic effects of our mutations were, however, on the inhibition of APC, where four orders of magnitude separated the best and worst inhibitors. We examined the structure of APC (Mather et al. 1996) for clues to the abrogating effect of the antithrombin RSL sequence on its inhibition. The established preference of APC for Arg at P1 and Pro at P2 is related to both the shape and the charge profile of the S1 and S2 pockets (Djie et al. 1996; Hermans and Stone 1993; Hopkins et al. 1995; Mather et al. 1996; Rezaie 1996). The bound serpin RSL presumably emerges in a westerly direction (in the "standard orientation") from these sites. The interaction between Gly216 [in the topologically based chymotrypsinogen numbering system (Bode and Huber 1992)] and the P3 main-chain interaction seen in canonical protease inhibitors (Bode and Huber 1991) is unhindered. The P3 side chain is not expected to make many direct contacts with the base of the reactive site cleft, however, the 5.6-fold increase in activity against APC caused by a P3 Ala-to-Ile mutation indicates that, in spite of this, the nature of the P3 side chain remains important and may make contact with the regions surrounding the active-site cleft, as demonstrated previously with E192Q variants of both thrombin and APC (LeBonniec and Esmon 1991; Rezaie and Esmon 1993). The residues that constitute the southern and southwestern flanks of the S region, Met175, Asn174, and Glu217, are good candidates for interactions with the P4 to P7 residues. It has been predicted that APC would have a preference for smaller side chains at the P4 site due to the presence of Met 175 at S4 (Bode et al. 1997), and indeed we observed a nearly threefold reduction in the reaction rate with APC when the P4 residue was mutated from an Ala to the larger Leu. The APC active-site cleft is "closed" in the west by a prominence defined in the south by both Asn174 and Met175 and in the north by Ser97 and Thr98; therefore the serpin peptide chain probably does not remain in direct contact with, and likely begins to turn away from, the surface of the protease at this point. In the thrombin–fibrinopeptide A complex, residue P6 (Glu11) makes close contacts in this area with Arg173, which is a serine in APC (Stubbs and Bode 1993). These contacts stabilize a turn reversing the direction of the peptide chain and may play a similar role with serpins. However, the P6 mutation performed here (Leu to Val) is conservative, and neither side chain would make, say, a hydrogen bond with the Ser173, which likely explains their small effect.

One of the most notable observations deriving from this investigation is illustrated in Fig. 2, which shows that the impact of individual residues on the rate of inhibition of APC may vary substantially. The effect of mutating the P2 residue from Pro to Gly on the rate of inhibition of APC varies by almost 250-fold, from a nearly 3-fold increase in reaction rate (LS7-3/P′2L to LS7-2′; p to k) to an 87-fold decrease (LS7-3′/P2P to LS7-3′; o to j). At position P2, proline instead of glycine will have restrictive effects on possible pathways taken by the bound serpin peptide chain and, consequently, affect the contribution of other residues to the stability of encounter and intermediate complexes. A P2 Pro might therefore be expected to reduce the impact of other residues on inhibition rates. However, there is no consistent direction in the effect of a P2 Pro versus Gly on the

impact of other mutations. The most substantial abrogation of the effect of the P3 to P7 residues is seen when comparing LS2-1 with LS7-1 (n and 1; which both have a glycine at P2), where only a 12-fold effect of the P3 to P7 residues is seen, compared to a 116-fold effect in the transition from LS2-3′ to LS7-3′ (e → j), both of which also have a Gly at P2 (Fig. 2). The difference between these two pairs of chimeras is the presence of the antithrombin P′2 Leu and P′3 Asn or the α_1-AT P′2 Ile and P′3 Pro.

Examination of the structures of thrombin, factor Xa, and APC shows that their S′3 sites are quite distinct. Relative to thrombin and factor Xa, the S′3 region of APC is open and large. Residue 39 of thrombin is a Glu, whereas the analogous residue of APC is a Lys; these residues have been shown to have important effects on the recognition of substrates at P′3 by both thrombin and APC, favoring those with charges opposite to that of residue 39 (Le Bonniec et al. 1991). There is, however, no apparent dependence of the effect of the P′3 Pro-to-Asn mutation on the charge of residue 39. The dependence of our P′3 Pro → Asn mutation on cooperativity for its large effect on the inhibition of APC, and the existence of some cooperativity with thrombin for the same residues, suggests independent properties of the RSL sequence. A more complex relationship may therefore exist between the P′3 Asn and the rate of inhibition of APC by antithrombin and our chimeras. One feature of the S′ region of APC that may have effects on its catalytic activity toward macromolecular substrates is Leu40; this residue protrudes into the area which would be occupied by the substrate, were it to follow the canonical path, and this will likely hinder the formation of canonical structures in substrates at this site (Bode et al. 1997; Mather et al. 1996). The presence of Pro at P′3 in our chimeras results in the apposition of two prolines at P′3 and P′4; the increased rigidity of such a structure may help overcome any hindrance that Leu40 poses to rapid catalytic attack.

We have extended the observed size of cooperative interactions in the determination of serpin specificity by an order of magnitude (*cf.* Chaillan-Huntington et al. 1997; Hopkins et al. 1995). It appears unlikely from our data that the cooperative effects observed are due to direct residue-to-residue contacts within the RSL, both for topological reasons and since there is a progressive decrease in the inhibition rate of APC as residues from P2 to P7 are substituted (in the presence of P′2 Leu and P′3 Asn), rather than an abrupt change caused by the presence of any particular residue. The cooperative effect observed between the P′3 Asn and the unprimed residues could be caused by long-range intraloop effects or by communication among protease sites as a consequence of the inhibitor binding to one or the other, or some combination of the two.

The flexible RSL of serpins is uniquely suited for communicating the information needed for cooperativity through conformational changes. It is likely that many serpins have RSL structures that do not readily fit into protease reactive-site clefts and, in some cases, are partly helical (Stein et al. 1990; Wei et al. 1994). Two separate crystal structures of the RSL of α_1-AT exist, within its natural setting (see Fig. 1) (Elliott et al. 1996) and grafted into antichymotrypsin (Wei et al. 1994). In the latter case, the structure is helical, and in the former, β-strand-like. The difference between the association rate constants of these two serpins for leukocyte elastase and chymotrypsin is up to 500-fold (Djie et al. 1997; Rubin et al. 1994), probably due at least in part to the greater ease with which the β-strand-like structure of wild-type α_1-AT would bind to the reactive site and undergo catalytic attack. The attack of the catalytic serine on the P1–P′1 bond is a central aspect of serpin inhibition (Olson et al. 1995; Stone and Le Bonniec 1997). These structures must adapt, at least temporarily, to a Michaelis-like structure; the rate of this adaptation will depend on both the RSL and the protease. Therefore, any perturbation in the rate of optimal positioning of the scissile bond, which is affected by both the serpin RSL structure and the protease active site cleft, will affect the overall rate of inhibition.

Many mutations have been made in thrombin and APC to explore the molecular basis of their specificities. In APC, Tyr99 is an important component of the S2 subsite; a Tyr99 → Thr mutation increases the susceptibility of APC to inhibition by antithrombin, possibly by as much as 300-fold—from $0.014 \times 10^1\ M^{-1}\ s^{-1}$ (Hermans and Stone 1993) to $4.2 \times 10^1\ M^{-1}\ s^{-1}$ (Rezaie 1996). The mutation of residue Glu192 to Gln in either thrombin or APC increases dramatically (by 100- and 700-fold, respectively) the rate of inhibition of these enzymes by wild-type (i.e., P1 Met) α_1-AT and, also, makes these proteases uncharacteristically susceptible to inhibition by the Kunitz inhibitor BPTI (LeBonniec and Esmon 1991; Rezaie and Esmon 1993, 1996). In a manner possibly analogous to the cooperation between P and P′ residues seen here, the E192Q mutation of both thrombin and APC overcomes their normal restrictions at both the P3 and the P′3 positions and also, in thrombin, to the P′2 position (Marque et al. 2000). The contribution of E192 to the specificity of thrombin and APC, while not yet completely attributed, appears to be related to both charge and allosteric effects (Marque et al. 2000; Rezaie and Esmon 1996; van de Locht et al. 1997). The structure of E192Q thrombin complexed with the Kunitz inhibitor BPTI (van de Locht et al. 1997) has shown that the 60's loop which forms part of the S3 and S2 subsites, and whose C-terminal portion abuts the S′ region of the catalytic cleft, is mobile and has an altered position as a result of BPTI binding. Alterations in the behavior of the 60's loop as a result of the E192Q mutation, as well as direct effects, may therefore contribute to the increased inhibition of thrombin E192Q by Kunitz inhibitors and by α_1-AT. Independently, the 60's loop has been shown

to play an important role in the inhibition of thrombin by serpins. Similar to the E192Q mutation, deletion of a portion of this loop (Pro60b–Pro60c–Trp60d) renders thrombin susceptible to inhibition by BPTI (Le Bonniec et al. 1993). In addition, this deletion has serpin-specific effects: in contrast to the E192Q mutation, it completely abolishes inhibition by wild-type α_1-AT and decreases the inhibition by antithrombin and P1-Arg α_1-AT by 100- and 50-fold, respectively, yet has no effect on inhibition by Protease Nexin I (Le Bonniec et al. 1995).

Facing the 60's loop from across the catalytic cleft is the "148 loop," a flexible region consisting of an insertion, relative to chymotrypsin, of six residues in thrombin and five residues in APC at position 148. Mutation of this loop in thrombin by deletion of Glu146, Thr147, and Trp148 has profound effects on thrombin's activity, both toward substrates and toward inhibition by serpins, both as a result of direct effects stemming from the loss of occlusion of the catalytic site and by modification of the S1 and S2 sites (Le Bonniec et al. 1994, 1995). Similarly to the above deletion in the 60's loop, this deletion abolished inhibition by wild-type α_1-AT and decreased the inhibition by antithrombin and P1-Arg α_1-AT by 350- and 60-fold, respectively. In contrast to the negligible effect of the 60's loop deletion on inhibition by Protease Nexin I, this deletion in the 148 loop decreased the inhibition by Protease Nexin I by over 1000-fold (Le Bonniec et al. 1995). In thrombin, these loops therefore demonstrate significant potential to be among the natural counterparts of RSL residues in the determination of inhibitory spectra, and likely are also so for APC.

The 60's and 148 loops of thrombin and APC are quite different. The 60's loop of thrombin has an insertion of eight amino acids relative to APC and the 148 loop of thrombin is one residue longer than that of APC and has a different sequence. Shen et al. (1999) deleted four residues from the 148 loop of APC (His144, Ser145, Ser146, and Arg147) which occupy a similar space to the above residues deleted from the thrombin 148 loop. In contrast to the deletions of thrombin's 148 loop, this deletion had no effect on the inhibition of APC by wild-type α_1-AT; inhibition by antithrombin was not examined.

The loops surrounding the active sites of thrombin and APC are clearly involved in determining their specificities and the spectrum of serpins that inhibit them. Intriguingly, many of the mutations in these loops in thrombin affect at once both S and S′ sites and, thus, possibly reflect the cooperative effects seen here between P and P′ residues in determining the inhibitory spectrum of the antithrombin RSL sequence toward APC. It is likely that there have evolved sequences in APC which are counterparts to the antithrombin RSL sequence and which also determine the poor reaction rate of antithrombin with APC. However, further consideration of specific possibilities in this regard related to the poor inhibition of APC by antithrombin and the chimeras described here necessarily awaits specific experimental data.

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