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Effect of reactive site loop elongation on the inhibitory activity of C1-inhibitor

Ineke G.A. Bos[a,*], Yvonne T.P. Lubbers[a], Eric Eldering[b], Jan Pieter Abrahams[c], C. Erik Hack[a,d]

[a] *Department of Immunopathology, Sanquin Research at CLB, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands*
[b] *Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, 1105AZ Amsterdam, The Netherlands*
[c] *Leiden Institute of Chemistry, Biophysical Structural Chemistry, 2333 CC Leiden, The Netherlands*
[d] *Department of Clinical Chemistry, VU Medical Center, 1081 HV Amsterdam, The Netherlands*

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Abstract

The serine protease inhibitor C1-Inhibitor (C1-Inh) inhibits several complement- and contact-system proteases, which play an important role in inflammation. C1-Inh has a short reactive site loop (RSL) compared to other serpins. RSL length determines the inhibitory activity of serpins. We investigated the effect of RSL elongation on inhibitory activity of C1-Inh by insertion of one or two alanine residues in the RSL. One of five mutants had an increased association rate with kallikrein, but was nevertheless a poor inhibitor because of a simultaneous high stoichiometry of inhibition (>10). The association rate of the other variants was lower than that of wild-type C1-Inh. These data suggest that the relatively weak inhibitory activity of C1-Inh is not the result of its short RSL. The short RSL of C1-Inh has, surprisingly, the optimal length for inhibition.

Keywords: C1-Inh; Complement; Serpin; Reactive site loop; Inhibition

1. Introduction

C1-Inhibitor (C1-Inh) is a *ser*ine *p*rotease *in*hibitor (serpin) that inhibits inflammation. Serpins are suicide inhibitors that function as a set mousetrap. The active serpin is in a metastable conformation with its flexible reactive site loop (RSL) protruding from the core of the molecule, free to interact with the target protease. The RSL contains the reactive site, P1–P1′, which is a pseudo-substrate for the target protease. A dramatic conformational change occurs in the serpin upon proteolytic attack. Before the protease can finish hydrolysis of the esterbond between its active site serine and the substrate, a movement of the RSL by 70 Å to the opposite side of the serpin molecule distorts the catalytic triad of the protease, covalently trapping the enzyme [1].

C1-Inh inhibits several target proteases such as activated C1r, C1s and MASPs of the complement system and factors XI, XII and kallikrein of the contact system. As such, it regulates the activity of several pathways of inflammation. Treatment with C1-Inh in inflammatory diseases like the vascular leakage syndrome, bone marrow transplantation, sepsis and acute myocardial infarction has led to inhibition of inflammation (reviewed by Caliezi et al. [2]), though large doses are required.

The inhibitory activity of C1-Inh is relatively weak compared to that of other serpins [3]. The reason for this at the molecular level is unknown, but for many other serpins, the inhibitory capacity was shown to be critically dependent on the length of the RSL (e.g. Ref. [4]). Based on a 3D-model and sequence alignment it is clear that the RSL of C1-Inh is two amino acids shorter than that of α1-antitrypsin, one amino acid before and one after the reactive site [5,6]. A study with α1-antitrypsin Pittsburgh variants with variable RSL length demonstrated that deletion of residues lowered the efficiency of inhibition and increased stability of the complex [4]. In the anticoagulant activation of antithrombin by heparin, the RSL is effectively length-

Abbreviations: C1-Inh, C1-Inhibitor; serpin, serine protease inhibitor; RSL, reactive site loop; P1, active site of serpin; SI, stoichiometry of inhibition; WT, wild-type

* Corresponding author. Tel.: +31-20-512-3171; fax: +31-20-512-3170.
E-mail address: i.bos@sanquin.nl (I.G.A. Bos).

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ened by expulsion of a few of its residues from the core of the structure, making the P1-Arg more reactive towards the target protease [7].

In this study we investigated whether elongation of the RSL of C1-Inh improved the inhibitory activity. We produced five variants with one or two alanine residues inserted in the RSL and investigated their inhibitory activity.

2. Materials and methods

2.1. Materials

C1-Inh cDNA in the expression vector for *P. pastoris*, products for cloning and expression, as well as reagents used for kinetic analysis and other interactions with target proteases have been described before [8]. Trypsin, soy bean trypsin inhibitor and phenyl methyl sulfonyl fluoride (PMSF) were obtained from Sigma Biochemicals (Steinheim, Germany) and human neutrophil elastase from Elastin products Company Inc. (Owensville, MO, USA). Benzamidine was obtained from Acros Organics (NJ, USA) and EDTA from Siegfried Handel AG (Zofingen, Germany).

2.2. Site-directed mutagenesis

Site-directed mutagenesis to generate insertion mutants was performed with splice-overlap PCR (modified from Ref. [9]). The site of insertion was chosen based on a sequence alignment with two other potent serpins, α1-antitrypsin and serpin K [6], and not chosen in the hinge region because that region is crucial for effective RSL insertion (Ref. [10] (reviewed by Davis [11,12]). The sequence of the sense-strand of the mutagenesis primers was ATCTCTGTGGCCCGCACAGCGCTGCTGGTCGCCTTTGAAGTGCAGCAG (for mutant P1′ aP4′ a), GCCATCTCTGTGGCCGCGCGCACCCTGCTGGTCT (P1a), CATCTCTGTGGCCCGTACGCTGCTGGTCGCCTTTGAAGTGCAGCAG CCC (P4′ a), TCCGCCATCTCTGTGGCCGCGCGCACCCTGCTGGTCGCCTTTGAAGTGCAGCAGCCC (P1aP4′ a), TCCGCCATCTCTGTGGCCGCGCGCACCGCCCTGCTGGTCTTTGAAGTG (P1aP1′ a). The flanking primers were designed on the pPICZαA vector to facilitate cloning of the entire gene in a pGEMT-easy vector. The DNA sequence was verified with sequence analysis (Applied Biosystems, 377XL platform). The RSL DNA was subcloned in the pPICZαA-C1Inh vector [8] via the restriction sites *Eco*RI (bp 1284) and *Not*I (in the multiple cloning site of the pichia vector, behind the C1-Inh gene). The five variants are shown in Table 1.

Table 1
Sequence of the reactive site between the Gly[431] and Pro[454] of C1-Inh RSL variants

Serpin	Sequence of the RSL part
C1-Inh WT	GVEAAAASAISVA **R**T LLV FEVQQP
C1-Inh P1′ aP4′ a	GVEAAAASAISVA **R**t**a**LLV**a**FEVQQP
C1-Inh P1a	GVEAAAASAISVA**a**RT LLV FEVQQP
C1-Inh P4′ a	GVEAAAASAISVA **R**T LLV**a**FEVQQP
C1-Inh P1aP4′ a	GVEAAAASAISVA**a**RT LLV**a**FEVQQP
C1-Inh P1aP1′ a	GVEAAAASAISVA**a**RT**a**LLV FEVQQP

The P1-Arg is depicted in bold, the inserted Ala residues are depicted in bold lower case.

2.3. Expression and purification of C1-Inh variants

The C1-Inh RSL variants were cloned and expressed in *P. pastoris* and purified from the supernatant as described before [8]. C1-Inh antigen was detected with ELISAs described before (summarised in Ref. [13]). Conformationally changed C1-Inh was determined with mAb Kok-12 [14] as the coating antibody in the same set-up as for antigen. Expression levels of the five C1-Inh RSL variants were similar to that of wild-type rhC1-Inh.

2.4. Kinetic analysis of C1-Inh RSL variants

Progress curves for the interaction of inhibiting C1-Inh RSL variants with C1s and coagulation factor XIIa under pseudo-first order conditions were performed as described before [8,15], with some minor modifications in C1-Inh concentration when the inhibitory activity was lower than for wild-type C1-Inh. The actual concentrations used can be deduced from Fig. 1. In control experiments, the association rate constant of plasma C1-Inh was determined and was similar to the constants described recently [8].

2.5. Stoichiometry of inhibition

Kallikrein (10 nM) or coagulation factor XIIa (100 nM) were incubated overnight with a 0.1–10-fold molar excess of purified C1-Inh. Residual proteolytic activity was measured by a 10-fold dilution in the chromogenic substrate S2302 (2.5 and 1.25 mM, respectively) and compared with a standard curve of kallikrein of XIIa. The stoichiometry of inhibition (SI) was defined as the abscissa on the *x*-axis.

2.6. Conformation of the C1-Inh RSL variants

Heat stability experiments were performed as described before [8], with the amount remaining antigen at 37 °C as the reference.

In proteolysis experiments with trypsin, prior to the determination of melting curves, 50 nM of C1-Inh was incubated with 5 nM of trypsin. To study the ability to interact with different proteases, the C1-Inh variants (20 nM) were incubated overnight with a 5-fold molar excess of the proteases C1s, kallikrein, factor XIIa, trypsin and human neutrophil elastase. Protease inhibitors (100 mM

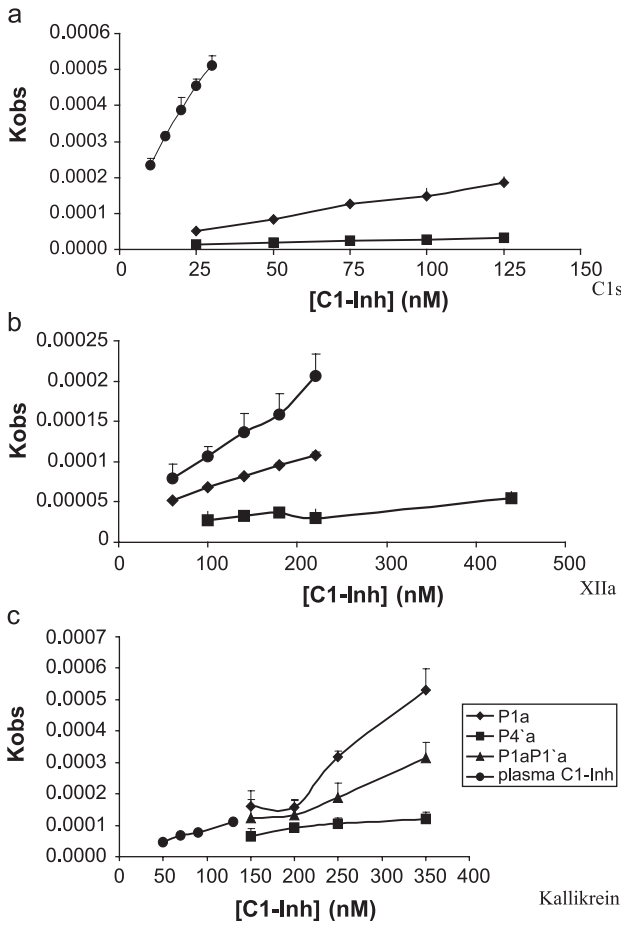

Fig. 1. Kinetic analysis of C1-Inh RSL variants. The k_{obs} was determined with the method of progress curves as described before [8] under pseudo-first order conditions with an excess of C1-Inh over target protease. This is the plot of k_{obs} versus [I] for the interaction of various C1-Inh RSL variants with C1s (a), factor XIIa (b), and kallikrein (c). k_{on} (Table 3) was calculated from this plot of k_{obs} versus [I] for the different RSL variants.

benzamidine, 10 mM EDTA, 0.1 mg/ml soy bean trypsin inhibitor and 2.5 mM PMSF) were added after the incubation and C1-Inh was subsequently analysed in a heat-stability experiment at 80 °C. The melting temperature, defined as the temperature at which 50% of the antigen remained in solution, was calculated according to the equation Ln ((% antigen − 100)/(0 − % antigen)) = slope × Ln Temp + intercept.

Table 2
Melting temperature of C1-Inh RSL variants

	Intact C1-Inh (°C)	Cleaved C1-Inh (°C)
C1-Inh WT	47	>80
C1-Inh P1′ aP4′ a	61	>80
C1-Inh P1a	54	>80
C1-Inh P4′ a	55	>80
C1-Inh P1aP4′ a	58	>80
C1-Inh P1aP1′ a	50	>80

C1-Inh in supernatants of *P. pastoris* was incubated for 2 h at different temperatures before (intact) or after (cleaved) incubation with trypsin. The levels of C1-Inh antigen remaining in the supernatant after centrifugation were measured with ELISA and expressed as percentage of levels of C1-Inh incubated at 37 °C. The melting temperature is the temperature at which 50% of the antigen remained in solution.

Table 3
Interaction of C1-Inh RSL with mAb Kok-12, specific for conformationally changed C1-Inh

Plasma C1-Inh	3%
C1-Inh P1′ aP4′ a	4%
C1-Inh P1a	3%
C1-Inh P4′ a	4%
C1-Inh P1aP4′ a	12%
C1-Inh P1aP1′ a	3%

The percentage of C1-Inh antigen reacting with this mAb is given in the table.

3. Results

3.1. C1-Inh RSL variants are in the active metastable conformation

Previously we showed C1-Inh can be inactivated by proteases in the *P. pastoris* expression system that we used to produce mutant protein [8]. Therefore we first investigated whether the C1-Inh RSL mutants where in the non-cleaved conformation. Gel electrophoresis is not well-suited to test proteolytic inactivation, as differential glycosylation of yeast C1-Inh smears out the protein in sizing gels. However, the unfolding profile of serpins at elevated temperatures is considered a good indicator of the labile tertiary structure that is characteristic for the native, metastable, serpin conformation [16,17]. Therefore we tested whether the C1-Inh variants that we produced were in the active metastable serpin conformation in heat-stability experiments. As depicted in Table 2, all RSL variants showed the characteristic heat-lability, although the melting temperature was higher than for wild-type C1-Inh. To investigate

Table 4
Association rate constants of the inhibiting RSL variants

	C1s	Factor XIIa	Kallikrein
	k_{on}* (M^{-1} s^{-1})	k_{on}* (M^{-1} s^{-1})	k_{on}* (M^{-1} s^{-1})
Plasma C1-Inh	$6.2(\pm 0.4) \times 10^4$	$4.5(\pm 0.3) \times 10^3$	$7.8\ (\pm 0.4) \times 10^3$
WT rhC1-Inh	$6.3(\pm 0.4) \times 10^4$	$5.7(\pm 0.2) \times 10^3$	$8.2\ (\pm 0.4) \times 10^3$
P1a	$5.2(\pm 0.8) \times 10^3$ *0.1 × WT*	$2.5(\pm 0.2) \times 10^3$ *0.5 × WT*	$1.19\ (\pm 0.06) \times 10^4$ *1.5 × WT*
P4′ a	$6.5(\pm 1.5) \times 10^2$ *0.01 × WT*	$5.6(\pm 0.6) \times 10^2$ *0.1 × WT*	$1.56\ (\pm 0.1) \times 10^3$ *0.25 × WT*
P1aP1′ a	N.D.	N.D.	$6.03\ (\pm 0.2) \times 10^3$ *0.75 × WT*

P1a,P4′ a, and P1aP1′ a as determined with the method of progress curves. The ratio of the association rate constant compared to WT rhC1-Inh is given in italics (0.1 × WT = association rate 10-fold slower than WT rhC1-Inh). The constants for plasma and recombinant wild-type C1-Inh were taken from Refs. [8,14,17].
N.D. = not determined.

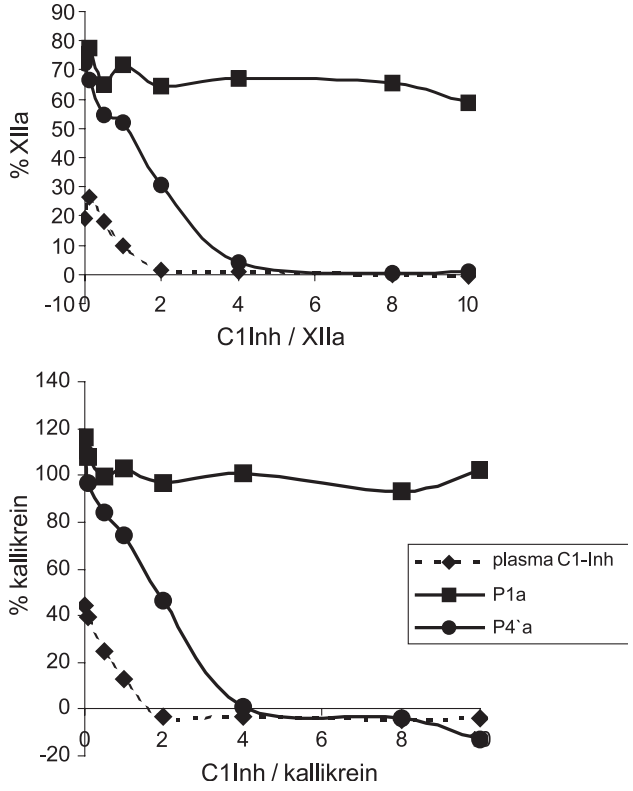

Fig. 2. Stoichiometry of inhibition of C1-Inh RSL variants with factor XIIa and kallikrein. The target proteases XIIa or kallikrein were incubated with an excess of C1-Inh. The ratio C1-Inh/target protease is depicted on the *x*-axis, the remaining protease activity (*y*-axis) was plotted against this ratio and the abscissa with the *x*-axis represents the SI.

whether this increase in heat-stability was due to formation of latent molecules, we studied the reactivity with mAb Kok-12. This mAb is specific for the changed conformation of C1-Inh. In Table 3, it is shown that the reactivity of the C1-Inh RSL mutants was hardly different from the reactivity with wild-type C1-Inh, except for mutant P1aP4′ a.

Trypsin cleaves C1-Inh at the P1−P1′ -peptidyl bond, converting the serpin to the stable, inactive conformation. The increased melting temperature after trypsin incubation (>80 °C) shows that all mutants had been converted to the stable cleaved conformation upon incubation with trypsin. This indicates that the RSL variants were in the typical metastable serpin conformation and were able to undergo the conformational change upon incubation with a protease.

3.2. Altered inhibitory activity of C1-Inh mutants towards target proteases

After pilot experiments with the C1-Inh variants in large molar excess over their target proteases (data not shown), selected combinations of mutant C1-Inh with their target protease were further investigated with the method of progress curves. All other combinations of C1-Inh mutant and target protease demonstrated no measurable inhibition in pilot experiments. Hence, kinetic constants of these mutants were not assessed. Association rate constants of target proteases with C1-Inh P1a, P4′ a, and P1aP1′ a were calculated from the plot of k_{obs} versus [C1-Inh] (Fig. 1) and are shown in Table 4. In general, the association rate was lower or at best comparable to that of wild-type C1-Inh. In one parameter, one of the mutants displayed more favorable characteristics than wild-type C1-Inh: the association rate constant of the P1a mutant for kallikrein was higher than that of wild-type C1-Inh. However, the concentrations required to achieve inhibition of target proteases by most mutants were higher than those of plasma C1-Inh (Fig. 1) were, due to dissociation of the complexes of C1-Inh with target protease. Finally, the SI for the interaction with factor

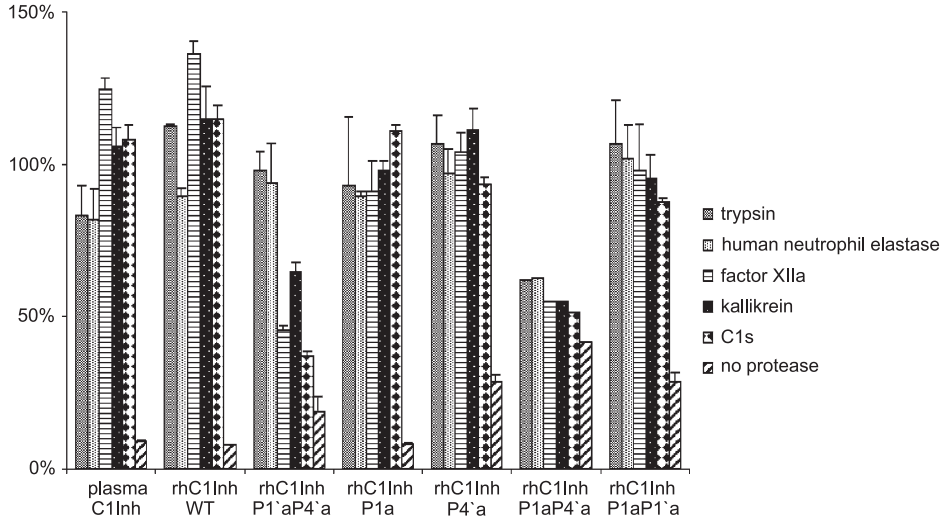

Fig. 3. Conformation of C1-Inh RSL variants after incubation with proteases. C1-Inh was incubated overnight with a 5-fold excess of trypsin, human neutrophil elastase, factor XIIa, kallikrein, or C1s. The conformation of C1-Inh was analysed in a heat-stability experiment. When the serpin has interacted with the target protease, the heat stability is high, resulting in high levels of remaining antigen at 80 °C (*y*-axis).

XIIa and kallikrein was determined (Fig. 2). Mutant P1a had a SI of >10, while mutant P4′ a had a SI of 4, only slightly higher than plasma C1-Inh (SI = 2). This indicates that the dissociation rate constant of these variants was increased, probably due to cleavage of the serpin.

The determined association rate constants may have been underestimated due to dissociation of protease–serpin complexes. This underestimation is negligible when SI~1, but becomes substantial when SI increases, like for the RSL mutants [3]. In mutant P1a, the association rate constant was increased 1.5-fold with a SI increase of >5. Therefore, the true association rate constant of this mutant with kallikrein is probably higher, supporting the hypothesis that RSL elongation enhances association.

3.3. The non-inhibiting C1-Inh variants, except for C1-Inh P1′ aP4′ a, are not inert to their target proteases

To investigate whether the non-inhibiting C1-Inh variants had been converted into a substrate for, or were inert to target proteases, the C1-Inh RSL mutants were incubated with a molar excess of target proteases. The conformation was studied by heat-stability at 80 °C (Fig. 3). All the C1-Inh mutants could be cleaved by trypsin and human neutrophil-elastase. C1-Inh P1′ aP4′ a did not react with C1s or factor XIIa and somewhat less with kallikrein as indicated by its heat-lability after incubation with these proteases. All other RSL insertion mutants reacted with all proteases, as they were heat-stable after incubation with these proteases. This suggested that these mutants were not inert to, but rather cleaved by the proteases.

4. Discussion

Zhou et al. [4] suggested that the RSL of serpins have an optimal length. This implies that elongation of the RSL of serpins with a relatively short RSL could possibly improve the inhibitory activity. Among the serpins with a relatively short RSL is C1-Inh. Here we demonstrate that the relatively poor inhibitory activity of C1-Inh towards its target proteases cannot be improved by lengthening its RSL to a size comparable to that of much more effective serpins like α1-antitrypsin. Only one of five mutants with elongated RSL had an increased association rate with kallikrein. However, this mutant nevertheless was a poor inhibitor because of a simultaneous increase in dissociation rate. The association rate of the other variants was lower than that of wild-type C1-Inh.

The observed decreases in association rate may be due to several factors including a non-ideal conformation of the RSL, which can diminish the rate of initial complex formation. Likely explanations for observed increased dissociation rates are less efficient trapping of the protease by mutants with an insertion before P1, since the peptide loop pulling the active site serine of the protease has become longer, or a slower insertion of the longer RSL of C1-Inh into the central β-sheet, facilitating cleavage of the serpin by the protease before actual trapping. Studies on a chimeric serpin of α1-antitrypsin with the RSL of the viral serpin Serp1 with and without four amino acids of α1-antichymotrypsin [18], revealed that insertion of four amino acids after the P1 resulted in a 10-fold slower association but the SI remained similar. Thus, placing the RSL in a non-ideal conformation can diminish the rate of initial complex formation. Our results are in agreement with this. The high SI supports this idea that inhibition is less efficient, not only because of less interaction with protease but also because of less efficient trapping.

The amino acid insertions in the RSL of C1-Inh were chosen based on the sequence of the RSL of α1-antitrypsin, which is two amino acids longer; one amino acid before and one after the P1. Insertion of amino acids before P1 might result in a shift of P1 or increased dissociation. For that reason, we investigated the effect of insertions both before and after P1. α1-Antitrypsin is a potent inhibitor of neutrophilic elastase [19]. One may postulate that the lack of activity of the mutants described in this article, may result from an inappropriate backbone of α1-antitrypsin as a serpin specific for trypsin-like proteases. This is not likely since the Pittsburgh mutant of α1-antitrypsin, which has arginine instead of methionine at the P1-position, is a potent inhibitor of thrombin [20], indicating that the RSL sequence of α1-antitrypsin may be adapted to trypsin-like proteases.

α1-Antitrypsin is able to trap protease tightly in spite of its longer RSL. A shorter central β-sheet of C1-Inh might explain this, but analysis of the Chou–Fassman parameters for β-strand propensity of the amino acids in the central β-sheet did not reveal a difference between C1-Inh and α1-antitrypsin (data not shown). A possible explanation for its short RSL is that C1-Inh has to interact with target proteases that have specific structural features.

Inhibition of factor XIa and C1s can be potentiated by heparin while inhibition of factor XIIa and kallikrein remains unaffected [21]. However, in RSL mutants P1a and P1aP1′ a of C1-Inh, we report different kinetics towards factor XIIa and kallikrein, indicating that the details of the inhibitory mechanism must differ for these proteases. This is also demonstrated in a recent study by De Taeye et al. [22]. This study shows a monoclonal antibody that had a stabilising effect on unstable PAI-1/t-PA complexes but a destabilising effect on unstable PAI-1/u-PA complexes.

The increased heat-stability of the RSL mutants suggests that the serpin might be in a loop-inserted conformation. However, analysis of the mutant proteins in an ELISA with monoclonal antibodies specific for this conformation (KOK12) [14] did not support this notion for most mutants. We proved in control experiments that the C1-Inh RSL variants were able to interact with Kok-12 after cleavage by trypsin, demonstrating that the lack of interaction is not due to a change of epitope in the variants (data not shown). Besides, no latent molecules were observed for mutants with

a three-amino acid insertion in the RSL of α1-antitrypsin [23]. Only mutant P1aP4′a might be partly in a latent conformation. This is supported by the fact that this mutant seems more or less inert to the target proteases C1s and XIIa, but on the other hand contradicted because this mutant can be cleaved by trypsin. We assume that a minority of these mutants is in a partly loop-inserted conformation.

Our data suggest that P1 exposure cannot be improved by RSL elongation, leading us to conclude that the short RSL length of C1-Inh is optimal for inhibition. At the evolutionary level, it can be hypothesized that natural selection has favored a slow inhibitor in order to maintain an efficient inflammatory reaction.

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