C1-inhibitor potentiation by glycosaminoglycans

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Chapter 5

Effect of reactive site loop elongation on inhibitory activity of C1-Inhibitor

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ABSTRACT

The serpin C1-Inhibitor (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 amino acids in the RSL. One of five mutants had an increased association rate with kallikrein, but was nevertheless 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. These data suggest that the short RSL of C1-Inh has the optimal length for inhibition.
Cl1-inhibitor (C1-Inh) is a SERine Protease INhibitor (serpin) that inhibits inflammation. Serpins are suicide inhibitors. The active serpin is in a metastable conformation with its flexible reactive site loop protruding from the core of the molecule, free to interact with the target protease. The reactive site loop (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 ester bond between its active site serine and the substrate, a movement of the reactive site loop 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 reactive site loop (e.g. ref 4). A study with α1-antitrypsin Pittsburgh variants with variable reactive site loop length demonstrated the effect of insertion or deletion of 1 or 2 residues before the reactive site. Addition of residues reduced the stability of the complex while deletion of residues lowered efficiency of inhibition and increased stability of the complex 4. In the anticoagulant activation of antithrombin by heparin, the RSL is effectively lengthened by expulsion of a few of its residues from the core of the structure, making the P1-Arg more reactive towards the target protease 5.

Based on a 3D-model and sequence alignment it is clear that the RSL of C1-Inh is 2 amino acids shorter than that of α1-antitrypsin, one amino acid before and one after the reactive site 6.7. Hence we investigated whether elongation of the reactive site loop of C1-Inh improved the inhibitory activity. We produced 5 variants with one or two amino acids inserted in the RSL and investigated their inhibitory activity.
MATERIALS AND METHODS

Materials
C1-Inhibitor 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. Trypsin was obtained from Sigma Biochemicals (Steinheim, Germany) and human neutrophil elastase from Elastin products Company Inc. (Owensville, MO, USA).

Site-directed mutagenesis
Site-directed mutagenesis to generate insertion mutants was performed with splice-overlap PCR (modified from 9). The site of insertion was chosen based on a sequence alignment with two other potent serpins, α1-antitrypsin and serpin K 7, and not chosen in the hinge region because that region is crucial for effective RSL insertion (reviewed by Davis 11, 12).

The sequence of the sense-strand of the mutagenesis primers was ATCTCTGTGGCCGCCACAGCCTGGTCTGCTGTCCCTTTGAAAGTGCAGCGG (for mutant P1’aP4’a), GCCATCTCTGTGGCCGCCGCGCACCCTGCTGGTCT (P1a), CATCTCTGTGGCCGCCGTCCTGCTGGTCCCGCTTTGAAAGTGCAGGCAGCC (P4’a), TCCGCCATCTCTGTGGCCGCCCGCGCACCCTGCTGGTCTTTGAAGTGCAGGCAGCC (P1aP4’a), and TCCGCCATCTCTGTGGCCGCCCGCGCACCCTGCTGGTCTTTGAAGTGCAGGCAGCC (P1aP4’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 reactive site loop DNA was subcloned in the pPICZαA-C1Inh vector via the restriction sites EcoRI (bp 1284) and NotI (in the multiple cloning site of the pichia vector, behind the C1-Inh gene). The 5 variants are shown in table I.

<table>
<thead>
<tr>
<th>Serpin</th>
<th>Sequence of the reactive site loop part</th>
</tr>
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<tbody>
<tr>
<td>C1-Inh WT</td>
<td>GVEAAASSAISVAaRT LLV FEVQPP</td>
</tr>
<tr>
<td>C1-Inh P1’aP4’a</td>
<td>GVEAAASSAISVAaRT LLV FEVQPP</td>
</tr>
<tr>
<td>C1-Inh P1a</td>
<td>GVEAAASSAISVAaRT LLV FEVQPP</td>
</tr>
<tr>
<td>C1-Inh P4’a</td>
<td>GVEAAASSAISVAaRT LLV FEVQPP</td>
</tr>
<tr>
<td>C1-Inh P1aP4’a</td>
<td>GVEAAASSAISVAaRT LLV FEVQPP</td>
</tr>
<tr>
<td>C1-Inh P1aP1’a</td>
<td>GVEAAASSAISVAaRT LLV FEVQPP</td>
</tr>
</tbody>
</table>

Table I
Sequence of the reactive site between the Gly431 and Pro454 of C1-Inh reactive site loop variants. The P1-Arg is depicted bold; the inserted Ala residues are depicted in bold lower case.
Expression and purification of Cl-Inh variants

The Cl-Inh RSL variants were cloned and expressed in P. pastoris and purified from the supernatant as described before. Cl-Inh antigen and active Cl-Inh were detected with ELISAs described before (summarised in 1). Expression levels of the 5 Cl-Inh RSL variants were similar to that of wild-type rhCl-Inh.

Kinetic analysis of Cl-Inh RSL variants

Progress curves for the interaction of inhibiting Cl-Inh RSL variants with C1s and coagulation factor XIIa under pseudo-first order conditions were performed as described before. In control experiments the association rate constant of plasma Cl-Inh was determined and was similar to the constants described recently.

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 Cl-Inh. Residual proteolytic activity was measured by a ten-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 was defined as the abscissa on the x-axis.

Conformation of the Cl-Inh RSL variants

Heat stability experiments were performed as described before, 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 Cl-Inh was incubated with 5 nM of trypsin. To study the interaction with different proteases, the Cl-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 and 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.

RESULTS

Cl-Inh reactive site loop variants are in the active metastable conformation

Previously we showed that Cl-Inh can be inactivated by proteases in the P. pastoris expression system that we used to produce mutant protein. Gel electrophoresis is not well suited to test proteolytic inactivation, as differential glycosylation of yeast Cl-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. Therefore we tested whether the Cl-Inh variants that we produced, were in the active metastable serpin conformation in heat-stability experiments. As depicted in table II all RSL variants
showed the characteristic heat-lability, although the melting temperature was higher than for wild-type C1-Inh. 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 reactive site loop variants were in the typical metastable serpin conformation and were able to undergo the conformational change upon incubation with a protease.

### Table II

<table>
<thead>
<tr>
<th>Melting temperature of C1-Inh RSL variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-Inh in supernatants of P. pastoris was incubated for 2 hours 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.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variant</th>
<th>Melting Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-Inh WT</td>
<td>47 °C</td>
</tr>
<tr>
<td>C1-Inh P1'ΔP4'a</td>
<td>61 °C</td>
</tr>
<tr>
<td>C1-Inh P1a</td>
<td>54 °C</td>
</tr>
<tr>
<td>C1-Inh P4'a</td>
<td>55 °C</td>
</tr>
<tr>
<td>C1-Inh P1aP4'a</td>
<td>58 °C</td>
</tr>
<tr>
<td>C1-Inh P1aP1'a</td>
<td>50 °C</td>
</tr>
<tr>
<td>C1-Inh</td>
<td>&gt; 80 °C</td>
</tr>
<tr>
<td>C1-Inh</td>
<td>&gt; 80 °C</td>
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<td>C1-Inh</td>
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<td>C1-Inh</td>
<td>&gt; 80 °C</td>
</tr>
</tbody>
</table>

**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. The plot of $K_{obs}$ versus [C1-Inh] (figure 1) was a straight line, implying a simple one-step reversible reaction. Association rate constants of target proteases with C1-Inh P1a, P4'a, and P1aP1'a are shown in table III. 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 favourable 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 (figure 1), due to dissociation of the complexes of C1-Inh with target protease. Finally, the stoichiometry of inhibition (SI) for the interaction with factor XIIa and kallikrein was determined. 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.
Figure 1
Kinetic analysis of C1-Inh RSL variants
The $K_{obs}$ was determined with the method of progress curves as described before. 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 III) was calculated from the plot of $K_{obs}$ versus $[I]$ for the different RSL variants.

Table III
Association rate constants ($k_{on}$, M$^{-1}$ s$^{-1}$) of the inhibiting reactive site loop variants 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 italic (0.1 * WT = association rate 10-fold slower than WT rhC1-Inh)

<table>
<thead>
<tr>
<th>Variant</th>
<th>C1s</th>
<th>Factor XIIa</th>
<th>Kallikrein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma C1-Inh</td>
<td>6.2(±0.4)*10^4</td>
<td>4.5(±0.3)*10^3</td>
<td>7.8(±0.4)*10^3</td>
</tr>
<tr>
<td>WT rhC1-Inh</td>
<td>6.3(±0.4)*10^4</td>
<td>5.7(±0.2)*10^3</td>
<td>8.2(±0.4)*10^3</td>
</tr>
<tr>
<td>P1a</td>
<td>5.2(±0.8)*10^3</td>
<td>2.5(±0.2)*10^3</td>
<td>1.19(±0.06)*10^6</td>
</tr>
<tr>
<td>P1aP1'a</td>
<td>2.5(±0.9)*10^3</td>
<td>1.5*WT</td>
<td>1.5*WT</td>
</tr>
<tr>
<td>P4'a</td>
<td>6.5(±1.5)*10^2</td>
<td>5.6(±0.6)*10^2</td>
<td>1.56(±0.1)*10^3</td>
</tr>
<tr>
<td>P1aP1'a</td>
<td>N.D.</td>
<td>N.D.</td>
<td>6.03(±0.2)*10^3</td>
</tr>
</tbody>
</table>

The constants for plasma and recombinant wild-type C1-Inh were taken from references 24 and 39.

N.D. = not determined
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\(^5\). 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.

*The non-inhibiting C1-Inh variants, except for C1-Inh P1\(^\prime\)aP4\(^\prime\)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 (figure 2). All the C1-Inh mutants could be cleaved by trypsin and human neutrophil-elastase. C1-Inh P1\(^\prime\)aP4\(^\prime\)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.

**Figure 2**

*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).
**DISCUSSION**

We could not increase the relatively poor inhibitory activity of C1-Inh towards its target proteases by lengthening its RSL to a size comparable to that of much more effective serpin inhibitors like α1-antitrypsin. However, one of five mutants had an increased association rate with kallikrein, but was nevertheless 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. 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-Fasman 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 by C1-Inh can be potentiated by heparin while inhibition of factor XIIa and kallikrein remains unaffected. 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 also differ for these proteases. 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 hypothesised that natural selection has favoured a slow inhibitor in order to maintain an efficient inflammatory reaction.

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**REFERENCE LIST**


