C1-inhibitor potentiation by glycosaminoglycans

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Chapter 7

Potentiation mechanism of C1-Inhibitor differs from that of other serpins

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ABSTRACT

Like a number of other serpins, C1-inhibitor (C1-Inh) is potentiated by heparin and other glycosaminoglycans. Here, we report that residues Lys^{284}, Arg^{367}, and Arg^{379} of C1-Inh, located close to the postulated secondary binding site for C1s, are important for potentiation. Known mechanisms of serpin potentiation are less likely to be involved in C1-Inh potentiation: (i) titration experiments did not support a template mechanism; (ii) stability of the central β-sheet was unaffected by heparin; (iii) no loop expulsion or P1 exposure was observed upon incubation with heparin; (iv) the major part of the unique N-terminal domain could be deleted without affecting potentiation. Together these data suggest a novel mechanism of serpin potentiation for C1-Inh, that is likely to involve residues close to C1-Inh’s secondary C1s binding site.
The serpin C1-Inhibitor (C1-Inh) is an inhibitor of several inflammatory cascades such as the classical pathway of complement and the contact system of coagulation. Because of its anti-inflammatory properties, administration of C1-Inh has been evaluated as a therapy of inflammatory conditions like the vascular leakage syndrome, bone marrow transplantation, sepsis and acute myocardial infarction (reviewed by Caliezi et al.). These studies revealed that high doses of C1-Inh are needed to induce an anti-inflammatory effect in vivo.

The inhibitory activity of several serpins including C1-Inh can be enhanced by heparin or other glycosaminoglycans (GAGs). For C1-Inh, the mechanism of potentiation by heparin is unknown. Administration of GAG-potentiated C1-Inh may help to reduce the dose of C1-Inh in inflammatory conditions. We have evaluated the use of C1-Inh potentiated by dextran sulphate in a rat model, and found that the non-covalent complex of C1-Inh and this artificial GAG is unstable in vivo, probably due to rapid dissociation and clearance of the GAG. A better understanding of the potentiation mechanism of C1-Inh by GAGs may aid in developing improved C1-Inh variants useful in a therapeutic setting.

Serpins like antithrombin III (ATIII), heparin cofactor II (HCII) and protein C inhibitor (PCI) also have enhanced inhibitory activity in the presence of GAGs. In general, potentiation results from two mechanisms. The template mechanism implies that the GAG molecule serves to assemble the protease and the serpin next to each other in an appropriate orientation. The second mechanism involves a conformational change in the serpin upon binding of the GAG making the inhibitor more reactive towards the protease.

The mechanism of potentiation of ATIII is well characterised, in particular since the complex of ATIII with a specific pentasaccharide has been crystallised. The heparin binding sites in ATIII are located in the A-, D- and P-helices, and in a cleft formed by the N-terminus. Upon binding of a core-pentasaccharide to the ATIII molecule, a small β-strand is pushed out of the central β-sheet, enhancing exposure of the reactive site. This leads to a 300-fold increase in inhibitory activity towards factor Xa. The potentiation towards thrombin requires at least 18 saccharides because a template mechanism is also involved. Since the reactive site loop (RSL) of C1-Inh is 5 amino acids shorter than that of ATIII, the mechanism of potentiation of C1-Inh by GAGs is unlikely to involve mobilisation of the RSL, and, therefore, must be different from that of ATIII.

The crystal structures of heparin cofactor II (HCII) and the Michaelis complex of HCII-Ser193→Ala thrombin suggest that GAG binding also induces expulsion of the RSL in HCII. Heparin binds to residues in the A- and the D-helix of HCII, which are conserved in ATIII and contribute to heparin binding. However, reactivity of HCII with thrombin requires also an intact N-terminus, even in the presence of GAGs. The structure of the N-terminal acidic tail has not been elucidated yet, but a mechanism involving allosteric activation has been proposed to explain involvement of this part of HCII in heparin binding. This includes expulsion of the RSL, closure of the central β-sheet, and consequently release of the acidic tail which is now free to recruit thrombin.
For C1-Inh it has been demonstrated that inhibition of target proteases is independent of the unique N-terminal domain \(^{20,21}\), but whether the N-terminal domain is involved in potentiation of C1-Inh by GAGs is unknown. The heparin binding regions of protein C inhibitor (PCI) are located in the A- and the H-helix, but only helix H-mutations result in altered rates of inhibition. The crystal structure of cleaved PCI suggests that more residues are involved in the positive electrostatic potential \(^{22}\) implying that the heparin-binding region may be larger than previously anticipated. Based on the surface electrostatic properties a co-occupation mechanism is proposed; PCI and activated protein C initially bind to different sites on heparin and then each migrates to form a complex through occupation of the same site on heparin.

Here we describe our studies on several possible mechanisms of potentiation of C1-Inh by heparin. Exposure of the reactive site loop, a classic template mechanism, conformational change of the central \(\beta\)-sheet, and involvement of the first 98 amino acids of the N-terminal domain could all be excluded. However, the positively charged residues Lys\(^{284}\), Arg\(^{287}\) and Arg\(^{378}\) close to the secondary Cls binding site were involved in potentiation.

**MATERIALS AND METHODS**

**Materials**

Plasma C1-Inh (Cetor) was obtained from Sanquin (Amsterdam, the Netherlands). Biotinylated polyclonal rabbit antibodies against human C1-Inh, and the monoclonal antibodies (mAb) RII (against human C1-Inh), and Kok12 (specific for cleaved or complexed human C1-Inh) have been described before \(^{23,25}\). Cls for ELISAs was purified and biotinylated (Cls-BT) as described before \(^{23}\). Streptavidin coupled to polymerised horseradish peroxidase (poly-HRP) was obtained from Sanquin (Dept. Immune Reagents). Streptavidin coupled to monomeric horseradish peroxidase was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Cls for kinetic assays was purchased from Calbiochem (La Jolla, CA, USA).

Heparin was purchased from Leo Pharma (Breda, the Netherlands). The kit to determine antithrombin activity (Coamic® antithrombin) and the chromogenic substrate S2314 were obtained from Chromogenix (Milano, Italy). The chromogenic assay for measurement of C1-Inh activity (Berichrom® C1-Inhibitor) was purchased from Dade Behring (Marburg, GmbH, Germany). C1-Inh N-terminal deletion mutants have been described before \(^{20}\). The mutants Lys\(^{284}\)→Gly, Arg\(^{287}\)→Gly, and Arg\(^{378}\)→Gly were produced with site-directed mutagenesis and expressed in \(P.\) pastoris, as described before \(^{26}\). Custom primers for site-directed mutagenesis were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA).
ELISAs
C1-Inh antigen, active C1-Inh, cleaved and inactive C1-Inh were determined as described before.\textsuperscript{22,27}

Peptidyl arginine deiminase treatment
These experiments were performed in a similar set-up as described before for ATIII\textsuperscript{14}: C1-Inh or ATIII (10 µM) were incubated with varying concentrations of peptidyl arginine deiminase (PADI) in 100 mM Tris-HCl, 5 mM CaCl\textsubscript{2}, pH 7.4, for 16 hours at 37°C in the presence or absence of heparin (50 µM). The reaction was stopped by addition of 50 mM EDTA. Residual inhibitory activity was tested with chromogenic assays for ATIII or C1-Inh activity.

Formation of multimers measured with dynamic light scattering
Multimer formation was assessed by incubation of C1-Inh (10 µM) for 15 minutes at 57°C in the presence or absence of heparin (50 µM). Protein size was determined by analysis of the particle radius with dynamic light scattering (DynaPro 99, Protein Solutions, NJ, USA).

Peptide insertion into the central β-sheet.
A custom peptide with the sequence N-acetyl-VEAAAAASISVAR, mimicking the sequence of the RSL, was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The peptide was dissolved at 1.1 mg per ml in water with 0.11 % acetonitril, 0.039 % NH\textsubscript{4}OH. C1-Inh (10 µM) in the presence or absence of heparin (50 µM) was incubated overnight at 37°C with varying amounts of the peptide (15 – 500 nM). The residual amount of active C1-Inh was determined with the ELISA for active C1-Inh.

Site-directed mutagenesis
Site-directed mutagenesis was used to generate mutants of C1-Inh. Mutagenesis was done by splice-overlap PCR (modified from\textsuperscript{28}). The sequence of the primers was ACA ACA TTT GAT CCC GGG AAA ACC AGA ATG GA for Lys\textsubscript{584}→Gly, GAT CCC AAG AAA ACC GGT ATG GAA CCC TTT CAC for Arg\textsubscript{287}→Gly and CTC CTA ACA CTA CCC GGG ATC AAA GTG ACG ACC for Arg\textsubscript{288}→Gly.

Potentiation by heparin
Potentiation of C1-Inh by heparin was studied by incubation of 2 nM C1s with 2 or 4 nM C1-Inh and 3.5 mM chromogenic substrate S2314 in the presence or absence of heparin. To analyse the possibility of a template mechanism, concentrations of 0.0125 – 1250 U per ml of heparin were used. To determine the potentiation of various C1-Inh mutants, a limiting concentration of heparin, i.e. 0.125 U per ml, was used. The amount of C1-Inh used in these experiments was determined by titrating C1-Inh (2, 4, and 8 nM) against active C1s (2 nM). It was determined in separate experiments that under these conditions, with a limiting heparin concentration, potentiation by heparin decreases the substrate conversion after 6 hours (data not shown). The effect of heparin on the activity
of the C1-Inh variants was subsequently determined at the C1-Inh concentration that resulted in inhibition similar to inhibition by plasma C1-Inh.

RESULTS AND DISCUSSION

Reactive site exposure of C1-Inh is not altered by heparin

Binding of heparin to ATIII results in an altered exposure of the reactive site residue arginin at the P1-position of the reactive site loop, as was deduced from enhanced sensitivity to peptidyl arginine deiminase (PADI) \(^\text{14}\). This enzyme cleaves off the imine group of the arginine at P1, thereby inactivating the serpin. Figure 1 shows ATIII and C1-Inh activity after incubation of the serpin with a varying amount of PADI. In the presence of heparin, lower concentrations of PADI were required to decrease ATIII activity. The K\(_m\) of the reaction of PADI with ATIII decreased in the presence of heparin (0.12 versus 0.032 \(\mu\)M PADI +/− heparin, respectively). The K\(_m\) of the reaction of PADI with C1-Inh was lower and irrespective of the presence of heparin (0.025 and 0.021 \(\mu\)M PADI +/− heparin, respectively). These results indicated that the exposure of the P1-Arg in the reactive site loop of C1-Inh was unaffected by heparin, and intrinsically more accessible than P1-Arg of ATIII.

It is therefore unlikely that potentiation of C1-Inh requires a substantial rearrangement of the reactive site loop. The observation that the RSL of C1-Inh is 5 amino acids shorter than that of ATIII gives further support against such a mechanism. In a separate study we have investigated the effect of RSL elongation on inhibitory activity, but this led to less effective inhibition (Bos et al., 2003, Chapter 5).

![Figure 1](image-url)

**Figure 1**

Heparin does not alter susceptibility of C1-Inh for PADI

C1-Inh or ATIII as a control (each at 10 \(\mu\)M) with and without heparin (50 \(\mu\)M) were incubated with an increasing amount of PADI (x-axis), which removes the imine-group from the active site, P1-Arg. Residual serpin activity was then measured with a chromogenic assay, and is depicted on the y-axis. Similar results were obtained in three separate experiments.
The conformation of the central β-sheet is not affected by heparin

Heparin can be postulated to induce a conformational change of C1-Inh, which results in an altered stability of the central β-sheet, as has been described for ATIII [14]. This mechanism was investigated in several ways. First, the effect of heparin on the heat stability of C1-Inh was analysed. As many other serpins, C1-Inh forms multimers at elevated temperatures as a result of insertion of the RSL of one molecule into the central β-sheet of another. An altered conformation of the central β-sheet probably induces a different stability at higher temperatures resulting in a change in the rate of multimer formation. A temperature of 57°C was chosen since in pilot experiments the size of multimers of normal plasma C1-Inh incubated for 2 hours at different temperatures increased in a linear fashion between 50 and 60°C (data not shown). C1-Inh multimerization at 57°C was unchanged in the presence of heparin (figure 2).

Another way to investigate the stability of the central β-sheet is by analysing the effect of insertion of a peptide mimicking the reactive site loop sequence into the central β-sheet. A peptide with the same sequence as the proximal part of the RSL was incubated with C1-Inh as described in materials and methods. No shift in dose-response curves of the percentage of active C1-Inh versus the peptide concentration occurred in the presence of heparin (figure 3). The Kd of the C1-Inh*peptide complex (0.12 and 0.15 mM in presence or absence of heparin), was irrespective of the presence of heparin. These data altogether suggested that the conformation of the central β-sheet was not affected by heparin.

Figure 2

Heparin does not alter multimer formation of C1-Inh

C1-Inh (10 μM) was incubated for 15 minutes at 57°C in the presence or absence of heparin (50 μM). During this period C1-Inh multimers were formed. Particle size of the multimers was measured with dynamic light scattering, the radius of the particles is depicted in the y-axis.
Heparin does not alter peptide insertion into the central β-sheet of C1-Inh

C1-Inh (10 µM) was incubated with a varying concentration of a peptide mimicking the reactive site loop sequence (x-axis) in presence or absence of heparin (50 µM). Remaining C1-Inh activity was measured with an ELISA for active C1-Inh and plotted on the y-axis. Similar results were obtained in 2 separate experiments.

Potentiation of C1-Inh by heparin is independent of the non-essential part of the unique N-terminal domain

Because the N-terminal domain of heparin cofactor II is probably involved in heparin binding and because C1-Inh also has a unique N-terminal domain, we analysed the potentiation of two N-terminal deletion mutants of C1-Inh. These two mutants start at amino acid 76 and 98 and have completely normal activity towards Cls, kallikrein and XIIa in vitro. This shows that the first 98 amino acids are not essential for inhibition. However, the two disulphide bridges between Cys76 and Cys98 and the serpin are essential to maintain the metastable conformation. For that reason we could not delete the entire N-terminal domain, which involves the first 115 amino acids. Table I shows the inhibition of Cls by C1-Inh mutants in the presence or absence of heparin. The effect of heparin on the N-terminal domain deletion mutants was similar to its effect on wild-type C1-Inh, hence this part of the N-terminus of C1-Inh is not involved in potentiation.
Table I

The major part of the unique N-terminal domain of C1-Inh is not involved in potentiation. C1s (2nM) with C1-Inh (2 or 4 nM) and 3.5 mM chromogenic substrate were incubated with or without a limiting amount of heparin (0.125 U/ml). Under these conditions C1-Inh potentiation leads to a decrease in substrate conversion. For each sample the absorption at 405 nm after 6 hours was expressed as a percentage of the absorption without C1-Inh. This percentage substrate conversion is depicted in the table.

<table>
<thead>
<tr>
<th>Substrate Conversion %</th>
<th>No heparin</th>
<th>Heparin 0.125 U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma C1-Inh</td>
<td>76 %</td>
<td>50 %</td>
</tr>
<tr>
<td>WT-rhC1-Inh</td>
<td>76 %</td>
<td>57 %</td>
</tr>
<tr>
<td>rhC1-Inh{76}</td>
<td>81 %</td>
<td>50 %</td>
</tr>
<tr>
<td>rhC1-Inh{98}</td>
<td>82 %</td>
<td>59 %</td>
</tr>
</tbody>
</table>

* C1-Inh{76} and C1-Inh{98} are deletion mutants starting at amino acid 76 and 98 respectively, thereby lacking the majority of the unique N-terminal domain. 20

Figure 4

A template mechanism is unlikely to dominate potentiation

C1s (2 nM) was incubated with 2 nM of C1-Inh, chromogenic substrate and increasing concentrations of heparin (x-axis). The substrate conversion after 6 hours is depicted by the A<sub>405</sub> on the y-axis. This experiment was performed twice with similar results.

A classic template mechanism can not explain potentiation

C1s can bind to heparin 29-31, although the literature is contradictory on this issue 32,33. Separate, independent binding of both serpin and protease is a hallmark of the classical template mechanism. A typical feature of the classical template mechanism as observed for AT and thrombin 34 is a bell-shaped curve when the concentration of GAG is plotted against the inhibitory activity of the serpin 35, as at higher heparin concentrations the serpin and the protease will bind to separate heparin molecules. When increasing heparin concentrations were tested, no bell-shaped curve was observed in the case of C1-Inh and C1s (figure 4), arguing against a template mechanism. The same results were obtained when another heparin preparation was tested (Celsius Laboratories, OH, USA). Involvement of some form of template mechanism can not be completely excluded because both C1s and C1-Inh seem to bind heparin. But based on these data, it is unlikely to play a dominant role in the enhanced inhibition.
Another characteristic of the classic template mechanism is the minimum length requirement of the heparin molecules. Low molecular weight dextran sulphate (5 kDa) is very effective in potentiation of C1-Inh, and moreover, in pilot experiments we observed potentiation by pentasaccharides (data not shown). Altogether these data indicate that a classic template mechanism cannot explain potentiation of C1-Inh by heparin.

Mutation of residues Lys^{284}, Arg^{287} and Arg^{278} of C1-Inh affects heparin-induced potentiation. C1-Inh activity can only be potentiated towards C1s and coagulation factor Xla, not towards coagulation factor XIIa and kallikrein. Hence involvement of a secondary protease-binding site in the potentiation mechanism can be postulated. Based on a 3D-model of C1-Inh we proposed 3 possible heparin-binding regions. Region 1 is located close to P1 and the secondary C1s binding site. Region 2 is located on the opposite side of P1 behind the hinge region, and region 3 is located around helix D. Region 3 is currently under investigation by other researchers. We did not find evidence of an AT-type potentiation, which would imply region 3, and because region 2 is too far from the C1s binding site we investigated region 1.

Region 1 comprises Lys^{284}, Arg^{287} and Arg^{278}. To evaluate a potential role of these residues in heparin-dependent potentiation of C1-Inh, they were mutated into glycine. Sequence analysis indicated that mutant Arg^{287}→Gly carried an additional mutation in the N-terminal domain: Pro^{76}→Leu. Since we had shown this part of the N-terminal domain not to be involved in inhibitory activity and heparin potentiation (table I) we analysed this double mutant instead. We confirmed these results with another double mutant of Arg^{287}→Gly, carrying the additional mutation Leu^{268}→Phe. The location of the possible heparin binding amino acids is depicted in figure 5a. Mutation of residues Lys^{284}, Arg^{287} or Arg^{278} in region 1 into glycine significantly impaired potentiation by heparin (figure 5b). Since single substitutions already showed an inhibitory effect on heparin potentiation, multiple residues appear to be directly involved. A combination of mutations might be required to fully block the effect of heparin. Based on the surface electrostatic potential we propose that residues Lys^{276}, Lys^{279} and Lys^{280} might also be involved.

The involvement of a secondary protease binding site is not unique, but also shown for thrombin, which interacts with the N-terminal acidic tail of HCII. A striking feature of heparin potentiation of C1-Inh is that it regulates the inhibitory activity towards some target-protases like C1s and factor Xla, but not others such as factor XIIa and kallikrein, postulating a role for the C1s binding site in potentiation.

The involvement of region 1 in heparin potentiation of other serpins has not been demonstrated so far, suggesting that this region is unique for C1-Inh. We confirmed this by analysis of the surface electrostatic potential of this region in the crystal structures of AT (1AZX, HCII (1JM), and PCI (1LQ8). None of these serpins showed a positively charged region at corresponding sites, indicating that involvement of region 1 in heparin-mediated potentiation is unique for C1-Inh.
The residues Lys284, Arg287, and Arg378 of C1-Inh are involved in heparin binding

a. Location of possible heparin binding residues in the 3D-structure

b. C1s (2nM) with plasma C1-Inh, rhC1-Inh, C1-Inh Lys284→Gly, C1-Inh Arg287→Gly or C1-Inh Arg378→Gly (4 nM) and 3.5 mM chromogenic substrate were incubated with increasing concentrations of heparin. Under these conditions C1-Inh potentiation leads to a decrease in substrate conversion. For each sample the absorption at 405 nm after 6 hours was expressed as a percentage of the absorption without C1-Inh. This is depicted on the y-axis. The experiment was repeated with a different substrate with similar results.
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