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Reformable intramolecular cross-linking of the N-terminal domain of heparin cofactor II

Effects on enzyme inhibition

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The crystal structure of a heparin cofactor II (HCII)–thrombin Michaelis complex has revealed extensive contacts encompassing the N-terminal domain of HCII and exosite I of the proteinase. In contrast, the location of the N-terminal extension in the uncomplexed inhibitor was unclear. Using a disulfide cross-linking strategy, we demonstrate that at least three different sites (positions 52, 54 and 68) within the N terminus may be tethered in a reformable manner to position 195 in the loop region between helix D and strand s2A of the HCII molecule, suggesting that the N-terminal domain may interact with the inhibitor scaffold in a permissive manner. Cross-linking of the N terminus to the HCII body does not strongly affect the inhibition of α-chymotrypsin, indicating that the reactive site loop sequences of the engineered inhibitor variants, required for interaction with one of the HCII target enzymes, are normally accessible. In contrast, intramolecular tethering of the N-terminal extension results in a drastic decrease of α-thrombin inhibitory activity, both in the presence and in the absence of glycosaminoglycans. Treatment with dithiothreitol and iodoacetamide restores activity towards α-thrombin, suggesting that release of the N terminus of HCII is an important component of the multistep interaction between the inhibitor and α-thrombin.

Keywords: α-thrombin; dermatan sulfate; heparin cofactor II; heparin; serpin(s).

Heparin cofactor II (HCII), a member of the serpin family, is an efficient inhibitor of α-thrombin in the presence of a variety of polyanions, including glycosaminoglycan (GAGs) such as heparin or dermatan sulfate [1]. In the absence of these compounds, the rate of α-thrombin inhibition is lowered by several orders of magnitude. HCII also inhibits α-chymotrypsin [2] and cathepsin G [3]; the reaction rates, however, are only moderately affected by GAGs. In recent years, substantial evidence has been accumulated on the molecular basis underlying the inhibition of serine proteinases by serpins [4,5]. Key features of the mechanism include the presentation of the inhibitor's reactive site loop (RSL) to a target enzyme, the initial cleavage of the scissile bond within the RSL, and formation of a covalent acyl ester intermediate between the catalytic serine of the enzyme and the carboxyl group of the P1 residue of the RSL. In the inhibitory path of the branched pathway mechanism of serpins, the RSL is inserted into β-sheet A with concomitant translocation of the attached proteinase to the opposite pole of the inhibitor [6,7].

For most serpins, the specificity of the inhibitor–enzyme reaction is primarily determined by residues within the RSL, with a dominant importance of residues flanking the scissile bond. However, exosite interactions can also play an important role, as recently demonstrated for the heparin-induced acceleration of inhibition of factors Xa and IXa by antithrombin [8]. Exosite contacts have also been implicated in the GAG-enhanced α-thrombin inhibition by HCII [9–11], and the crystal structure of S195A α-thrombin-complexed HCII has revealed extensive interactions that include sandwiching of the inhibitor's N-terminal domain between the serpin body and α-thrombin [12], which are distinct from the classical RSL/active site cleft inhibitor–enzyme interactions. However, it was not possible to locate the unique N-terminal extension, with its imperfect tandem repeat enriched in acidic amino acids [13,14], in the uncomplexed HCII molecule.

The accelerating effect of different GAGs on α-thrombin inhibition by HCII includes a complex series of processes, the relative importance of which may depend on the nature of the activating polyanion. Heparin and dermatan sulfate have been suggested to act as a template for surface approximation of enzyme and inhibitor [15,16]. Other work suggests that GAGs may liberate the acidic N-terminal domain of HCII from intramolecular interactions by displacement, providing an exosite for binding to α-thrombin [9,10,17], eventually in combination with a bridging mechanism [1,17,18]. Based on the results of X-ray crystallography, binding of GAGs to HCII has been proposed to initiate allosteric changes that include expulsion of the RSL,

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Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; RSL, reactive site loop; SI, stoichiometry of inhibition; wt-rHCII, wild-type recombinant heparin cofactor II.

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closure of β-sheet A and release of the acidic N-terminal tail for interaction with α-thrombin [12]. However, the location and function of the elusive N-terminal domain in the uncomplexed inhibitor molecule remained unclear.

Materials and methods

Materials

COS-7 cells and Chinese hamster ovary (CHO) DUKX B1 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Lipofect-AMINE PLUS™ and media were purchased from Life Technologies. A peroxidase-coupled donkey anti-rabbit IgG, HiTrap heparin HP columns, HiTrap steptavidin HP columns, Q-Sepharose-FastFlow, StreamLine rProtein-A agarose, poly(vinylidene difluoride) Hybond-P membranes, and Hyperfilm ECL films were from Amersham Biosciences. Human α-thrombin (> 3030 NIH units·mg^{-1}), α-chymotrypsin from human pancreas, dermatan sulfate from porcine intestinal mucosa (36 000 M_r), heparin from porcine intestinal mucosa (12 500 M_r, 181 USP units·mg^{-1}), polyethylene glycol (8000 M_r), dithiothreitol, reduced and oxidized glutathione, and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were purchased from Sigma. *N*-tosyl-Gly-Pro-Arg-*p*-nitroanilide was from Roche Diagnostics (Mannheim, Germany). H-D-Phe-Pro-Arg-chloromethyl ketone hydrochloride was from Bachem Biochemica GmbH (Heidelberg, Germany). Cu(II)-dichloro(1,10-phenanthroline) was from Aldrich. The expression vector pcDNA3.1(+) was purchased from Invitrogen. G418 sulphate was obtained from PAA Laboratories (Linz, Austria).

Construction, characterization and expression of HCII variants

HCII cDNA variants were constructed using either a variant of the megaprimer PCR method [19] or by overlap extension PCR mutagenesis [20]. The mutagenized DNA fragments were subcloned into the pPCR-Script™ Amp cloning vector (Stratagene). Appropriate restriction fragments of wild-type (wt)-HCII cDNA inserted into the expression vector pCDM8 [9] were then replaced by the corresponding genetically engineered variant areas, and the identity of all variants (Table 1) was verified by DNA sequencing. For initial characterization of the mutants, the medium of transiently transfected COS-7 cells was examined by Western blot analysis for the presence of HCII immunoreactive material and the ability to form SDS stable HCII–α-thrombin complexes.

For stable expression in CHO cells, 1.6 kb *Eco*RI cDNA fragments, coding for HCII variants, were excised from pCDM8 and cloned into the *Eco*RI site of pcDNA3.1(+), which contains a neomycin-resistance gene for selection. Transfection of CHO DUKX B1 cells with *Sca*I-linearized expression plasmids was performed by lipofection in serum-free Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (1 : 1, v/v). Selection in DMEM/Ham's F12 medium supplemented with 10% (w/v) fetal bovine serum and G418 sulphate (600 μg·mL^{-1}) was started after 2 days, and drug-resistant cells were expanded. HCII levels in the medium were monitored immunologically [21]. Cell lines secreting ≈ 0.4–3.2 μg of HCII per 10^6 cells each day were selected and cultured in serum-free DMEM for further investigation.

Table 1. Recombinant heparin cofactor II (HCII) variants investigated in this study. wt-rHCII, wild-type recombinant heparin cofactor II.

Variant	Sequence
wt-rHCII	P52, G54, S68, F195, C273, C323, C467
ΔC	P52, G54, S68, F195, C273S, C323S, C467S
ΔC/F195C	P52, G54, S68, F195C, C273S, C323S, C467S
ΔC/P52C/F195C	P52C, G54, S68, F195C, C273S, C323S, C467S
ΔC/G54C/F195C	P52, G54C, S68, F195C, C273S, C323S, C467S
ΔC/S68C/F195C	P52, G54, S68C, F195C, C273S, C323S, C467S

Preparation of immunoaffinity columns and purification of recombinant HCII variants

Human HCII from outdated plasma was isolated, as described previously [21], and biotinylated via the oligosaccharide chains by using EZ-Link™ Biotin-LC-Hydrazide (Pierce Biotechnology), as suggested by the supplier. The modified protein was purified using size-exclusion chromatography (Superdex 75 HR 10/30; Amersham Biosciences), adhered on a HiTrap streptavidin HP column (1 mL bed volume), and used for affinity purification of anti-HCII Ig. To achieve this, 2 mL of rabbit anti-human HCII IgG [9] was diluted 1 : 1 (v/v) with TAES (30 mM Tris/HCl, 20 mM sodium acetate, 1 mM EDTA, 0.5 M NaCl, pH 8.0), and after addition of phenylmethanesulfonyl fluoride (final concentraion 1 mM), the serum was applied to the matrix-bound HCII (flow rate: 152 cm·h^{-1}), pre-equilibrated in TAES. After washing with five volumes of TAES, bound antibodies were eluted with five volumes of Gentle Ag/Ab Elution Buffer (Pierce Biotechnology), dialysed (three times, 0.5 L each) against 0.2 M sodium borate, 0.5 M NaCl, pH 8.5, at 4 °C for 12 h, and concentrated using a Microsep concentrator (30 000 M_r; Pall Life Sciences). Antibody purity was evaluated by Coomassie Brilliant Blue staining following SDS/PAGE.

Coupling of purified immunoglobulins to solid support was performed essentially as described previously [22,23]. Anti-human HCII Ig (3 mg in 10 mL) were incubated for 1 h at room temperature in a suspension (5 mL) of StreamLine rProtein-A agarose pre-equilibrated in 0.2 M sodium borate, 0.5 M NaCl, pH 8.5. The beads were washed twice with 0.2 M sodium borate, pH 9.0, and resuspended in the same buffer (10 mL each). Bound immunoglobulins were covalently linked to the matrix by adding solid dimethyl pimelinediimidate dihydrochloride (Fluka, Deisenhofen, Germany) to a final concentration of 20 mM. After 1 h at room temperature, residual coupling reagent was inactivated by a 2 h incubation period at room temperature in 10 mL of 0.2 M ethanolamine, pH 8.0. Coupling efficiency was determined by SDS/PAGE. The antibody matrix was stored at 4 °C in a buffer comprising 50 mM Tris/HCl, 0.15 M NaCl, 0.02% NaN_3, pH 7.4.

Recombinant CHO cells producing HCII variants were cultured to reach an almost confluent state, and after

4 days in serum-free DMEM containing bovine insulin (10 $\mu g \cdot mL^{-1}$) and human transferrin (10 $\mu g \cdot mL^{-1}$), the medium (0.6–1 L) was collected and centrifuged. All further steps were carried out at 4 °C. After dialysis against 20 mM Tris/HCl, 1 mM EDTA, pH 8.0, the supernatants were filtered and applied to a Q-Sepharose FastFlow column (25 mL bed volume, flow rate: 120 $cm \cdot h^{-1}$). After washing (three column volumes), proteins were concentrated by elution with 40 mL of 20 mM Tris/HCl, 0.5 M NaCl, 1 mM EDTA, pH 8.0. The eluates were diluted with a 0.1 volume of Gentle Ag/Ab Binding Buffer and mixed under slight shaking (1 h) with the anti-HCII Ig resin (5 mL) pre-equilibrated in the same buffer. The resin was washed three times with binding buffer, and bound proteins were eluted with Gentle Ag/Ab Elution Buffer in four subsequent steps (total volume: 20 mL). After dialysis against 3×2 L of buffer (20 mM Tris, 150 mM NaCl, 1 $g \cdot L^{-1}$ PEG 8000, pH 7.4) at 4 °C for 12 h, the proteins were ultrafiltrated in Centriprep-10 concentrators (Millipore). Protein concentration was determined for each HCII variant by using an individually calculated extinction coefficient for the absorbance at 280 nm (Peptide Property Calculator, Center for Biotechnology North-western University, Evanston, IL, USA). The purity of the variants was determined by SDS/PAGE.

To determine the relative heparin affinity, supernatants from transfected cells were dialyzed against 20 mM Tris/HCl, 1 mM EDTA, pH 7.4, and fractionated on a HiTrap heparin column, in the presence or absence of 3 mM dithiothreitol, using a linear NaCl gradient (0–1 M). The NaCl concentration was determined by on-line conductivity monitoring. Fractions of 2 mL were collected and assayed for HCII by using a sandwich-type ELISA [21].

Reduction and reoxidation of disulfide bridge-containing variants *in vitro*

Serum-free medium from CHO cells secreting HCII variants containing a cysteine pair was adjusted to pH 8.0, incubated for 1 h at room temperature in the presence of 2 mM dithiothreitol, and dialyzed twice against 50 mM Tris/HCl, 150 mM NaCl, pH 8.0, for 6 h at 4 °C. Reoxidation was performed overnight with a mixture containing 2 mM reduced and 1 mM oxidized glutathione [24] at room temperature or in the presence of 50 μM Cu(II)-dichloro (1,10-phenanthroline) [25] at 4 °C, respectively.

Cyanogen bromide (CNBr) cleavage

Supernatants from transfected COS-7 cells were dialyzed against 20 mM Tris/HCl, 1 mM EDTA, pH 7.4, and purified partially on a HiTrap heparin column, as described above. Fractions containing HCII variants were pooled, concentrated by ultrafiltration and adjusted to 20 mM Tris/HCl, pH 7.4. Fifty microlitre aliquots containing ≈ 0.2 μg of HCII were degassed, and after addition of 125 μL of nitrogen-saturated formic acid (99%), the mixture was incubated with CNBr (2%, w/v) for 60 h at room temperature in the dark [26]. Excess reagent was removed by two cycles of lyophilization. The fragments were dissolved in twofold concentrated Laemmli sample buffer, lacking reducing agent, and then split into two aliquots. 2-Mercaptoethanol was added to one aliquot of each sample [final concentration: 5% (v/v)]. After SDS/PAGE (14% gels) the samples were analyzed by Western blotting for immunoreactive HCII fragments.

SDS/PAGE and Western blot analysis

After addition of one volume of twofold concentrated Laemmli sample buffer (with or without 5% 2-mercaptoethanol), the protein samples were heated, fractionated by SDS/PAGE in Tris/glycine/SDS running buffer and transferred to poly(vinylidene difluoride) membranes. After treatment with $NaCl/P_i$ (PBS) containing 3% bovine serum albumin and 0.3% Tween-20 (v/v) at 4 °C overnight, the membranes were incubated (1 h at room temperature) with an anti-HCII rabbit IgG (1 : 20 000 dilution). After washing, a peroxidase-coupled donkey anti-rabbit IgG (1 : 2000 dilution) was added (for 1 h), and HCII immunoreactive material was identified by exposure on Hyperfilm ECL films.

Enzyme assays and determination of inhibition rate constants of recombinant HCII variants

Active-site titration of α-thrombin was performed in 20 mM Tris/HCl, 150 mM NaCl, 0.1% PEG 8000, pH 7.4, using the irreversible inhibitor H-D-Phe-Pro-Arg-chloromethylketone. The enzyme was mixed with various amounts of the inhibitor and incubated at room temperature for 60 min. Residual activitiy was determined from the hydrolysis of 500 μL of the chromogenic substrate *N-p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide (150 μM). The concentration of active enzyme, E_0, was obtained from nonlinear regression analysis using the following equation:

$$v = SA(E_0 - 0.5\{(E_0 + I + K_i) - [(E_0 + I + K_i)^2 - 4E_0I]^{\frac{1}{2}}\}),$$

where v, percentage residual activity; SA, specific activity; E_0, enzyme concentration; and I, inhibitor concentration [27]. Stoichiometry of inhibition (SI) values were evaluated by incubation (90 min) of α-thrombin (2 nM) with various amounts of HCII variants in the presence of 10 $U \cdot mL^{-1}$ heparin or 100 $\mu g \cdot mL^{-1}$ dermatan sulfate, essentially as described previously [28].

Second-order rate constants (k_2) were determined under pseudo first-order conditions [17,28], using wild-type recombinant HCII (wt-rHCII) or variants purified by immune affinity chromatography. To determine α-thrombin inhibition rates of the reduced forms of variants with a cysteine pair, disulfide bonds were resolved with dithiothreitol, followed by treatment with iodoacetamide [29] and dialysis. Controls lacking an internal disulfide bridge were treated accordingly.

Purified HCII variants (50–250 nM) were incubated at room temperature in disposable polypropylene cuvettes with α-thrombin (5–10 nM) or α-chymotrypsin (25 nM) in 20 mM Tris/HCl, 150 mM NaCl and 0.1% (w/v) PEG 8000, pH 7.4. GAGs were used at concentrations of 10 $U \cdot mL^{-1}$ (heparin) or 100 $\mu g \cdot mL^{-1}$ (dermatan sulfate), respectively, unless stated otherwise. Dermatan sulfate was treated with sodium nitrite in acetic acid and dialysed prior to use [30]. Reactions were initiated by addition of the enzymes and

terminated after variable time-periods of incubation (15 s to 120 min) with 500 μL (final concentration 150 μM) of the appropriate chromogenic substrate (*N*-*p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide for α-thrombin or *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for α-chymotrypsin) and residual enzyme activity was monitored at 405 nm. Second-order rate constants were calculated from linear regression analysis of eight to 16 independent reactions, according to a previously published equation [10].

Results

Design of mutants

Human HCII contains three cysteine residues at positions 273, 323 and 467, respectively [13,14]. The sulfhydryl-containing amino acids do not play a major role in the heparin-enhanced α-thrombin inhibitory activity of HCII, nor are they involved in disulfide bridge formation [12,31]. Therefore, HCII variants were designed on a cysteine-free background in order to avoid problems with folding during the biosynthesis of the inhibitor molecules. The structures of several crystallized serpins (including that of HCII) indicated that residues in the loop connecting strand s2A and the GAG-binding helix D are surface exposed (Fig. 1). As a potential anchor site for interaction with the N-terminal domain, position 195 in this loop region was chosen. Pro52, Gly54 (located at the N-terminal end of the first acidic repeat) and Ser68 (located at the N-terminal side of the second acidic repeat) were selected as possible interaction partners for position 195. Table 1 summarizes the sequences of the variants examined.

Analysis of reformable disulfide bond formation

SDS/PAGE, under reducing and nonreducing conditions, respectively, may be used to monitor formation of intramolecular disulfide bonds [32]. To examine the effect of 2-mercaptoethanol on the electrophoretic mobility, wt-rHCII, a variant devoid of cysteine residues (ΔC), and mutants harboring one (ΔC/F195C) or two (ΔC/P52C/F195C, ΔC/G54C/F195C, ΔC/S68C/F195C) cysteine residues were expressed in COS-7 and CHO cells. Supernatants from cells cultured in serum-free medium were diluted with an equal volume of twofold concentrated Laemmli sample buffer, containing or lacking 2-mercaptoethanol, and fractionated by SDS/PAGE. All variants depicted identical electrophoretic mobilities (≈ 76 000 M_r) after reduction (Fig. 2A). In contrast, all mutants containing an engineered pair of cysteine residues migrated faster in the absence of reducing agent compared with controls containing no, or only one, cysteine residue (Fig. 2B), indicating the formation of intramolecular disulfides. The extent of the mobility shift depended on the position of the cysteine residue in the N-terminal domain. HCII oligomers were not observed, and dimers were detected only after film overexposure, demonstrating that disulfide formation in the monomer is strongly preferred (not shown).

The presence of intramolecular disulfide bridges was also investigated by analysis of the CNBr cleavage fragments. Treatment of wt-rHCII with CNBr was expected to generate 19 peptides (Table 2). Formation of a disulfide bond between position 52, 54 or 68, and position 195, should connect the two largest CNBr cleavage peptides that are expected to be split to the original peptides after

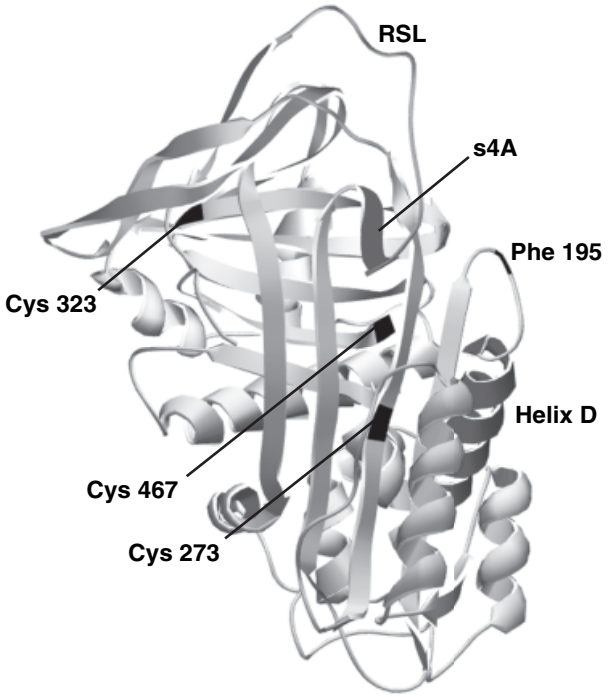

Fig. 1. Three dimensional structure of heparin cofactor II (HCII) (chain A, positions 95–480; PDB entry: 1JMJ). RSL, reactive site loop.

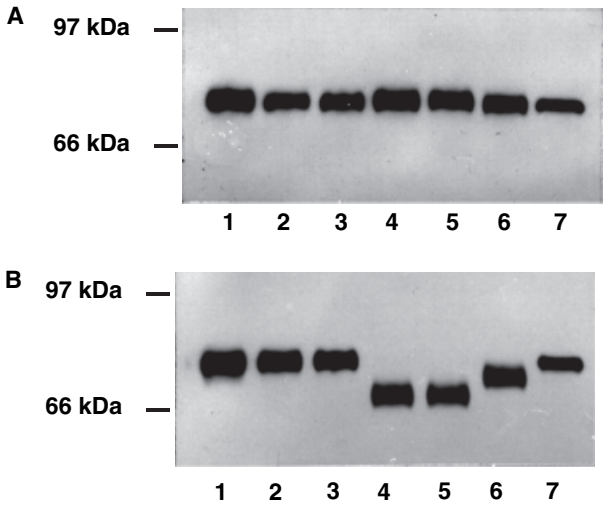

Fig. 2. Western blot analysis of wild-type recombinant heparin cofactor II (wt-rHCII) and engineered variants under reducing or nonreducing conditions. Prior to SDS/PAGE (10% gels), the samples were incubated in Laemmli sample buffer for 3 min at 95 °C in the presence (A) or absence (B) of 2-mercaptoethanol. Lane 1, wt-rHCII; lane 2, variant ΔC; lane 3, variant ΔC/F195C; lane 4, variant ΔC/P52C/F195C; lane 5, variant ΔC/G54C/F195C; lane 6, variant ΔC/S68C/F195C; lane 7, HCII from human plasma. The positions and sizes of marker proteins are indicated on the left.

Table 2. Cyanogen bromide (CNBr) cleavage fragments of heparin cofactor II (HCII). Formation of an intramolecular disulfide between the N-terminal domain (position 52, 54 or 68, respectively) and position 195 results in linkage of fragments no. 2 and no. 4.

Fragment no.	CNBr cleavage site (amino acid position[a])	Fragment size (amino acids)	Calculated mass of fragment[b] (M_r)
1	33	33	3521[c]
2	143	110	12 394[d]
3	145	2	158
4	248	103	12 169[c]
5	268	20	2150
6	269	1	101
7	288	19	2239
8	306	18	2161
9	307	1	101
10	336	29	3137
11	344	8	887
12	347	3	245
13	366	19	2251
14	393	27	3228[c]
15	397	4	427
16	405	8	889
17	442	37	3901
18	471	29	3481
19	480 (C terminus)	9	943

[a] Numbering refers to position 1 of mature HCII [9,13]. [b] Post-translational modifications are not taken into account. [c] N-glycosylated fragment. [d] Peptide contains two sulfated tyrosine residues.

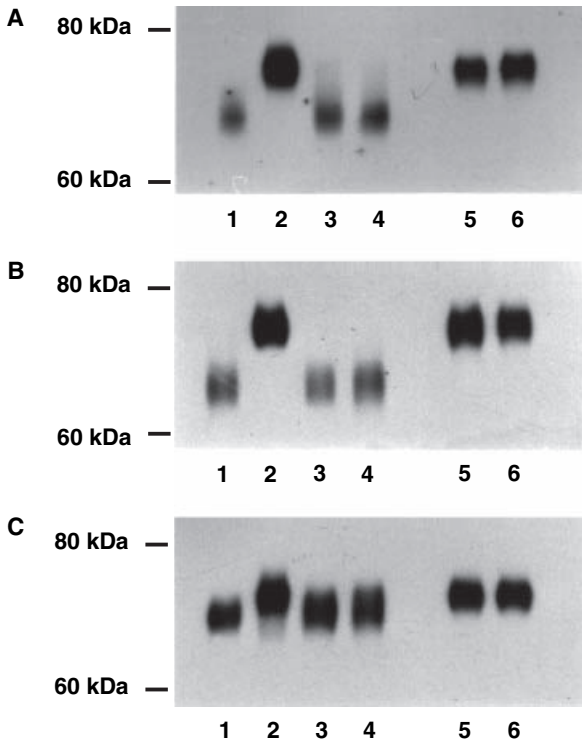

Fig. 3. Reoxidation of heparin cofactor II (HCII) variants ΔC/P52C/F195C (A), ΔC/G54C/F195C (B) and ΔC/S68C/F195C (C) *in vitro*. The supernatants from recombinant Chinese hamster ovary (CHO) cells coding for HCII variants with intramolecular disulfide bonds (lanes 1) were reduced and dialyzed (lanes 2), and exposed to ambient oxygen in the presence of 50 μM Cu(II)-phenanthroline (lanes 3) or treated with a mixture containing 2 mM reduced and 1 mM oxidized glutathione (lanes 4). Lanes 5 and 6 show 2-mercaptoethanol-treated aliquots of the reoxidized material depicted in lanes 3 and 4, respectively. After nonreducing SDS/PAGE and Western blotting, HCII variants were detected immunologically. The sizes and positions of the marker proteins are indicated.

treatment with dithiothreitol. Western blot analysis of the reduced fragments from CNBr-treated variants with cysteine pairs, and from wt-rHCII, revealed two closely spaced bands (≈ 21 000 M_r and ≈23 000 M_r) as the only signals. The same pattern was observed for unreduced wt-rHCII. In contrast, a single immunoreactive fragment with decreased mobility (≈ 34 000 M_r) was detected with the unreduced variants containing a pair of cysteine residues, indicating linkage of fragments no. 2 and no. 4 (data not shown).

To exclude the possibility that the N-terminal region had been forced to interact with position 195 as a result of misfolding within cells, the disulfide-containing HCII variants were reduced with dithiothreitol, dialyzed and then exposed either to ambient oxygen in the presence of Cu(II)-phenanthroline or to a mixture of reduced and oxidized glutathione, respectively. SDS/PAGE revealed that all three double-cysteine variants may be reconverted nearly quantitatively into the disulfide-containing forms (Fig. 3). Treatment of the reoxidized samples with 2-mercaptoethanol confirmed that the mobility shift observed after reoxidation was not caused by proteolytic degradation.

Effects of intramolecular disulfides on heparin affinity

To examine whether tethering of the N-terminus to position 195 influences heparin affinity, wt-rHCII and variants containing a pair of cysteine residues were fractionated on a HiTrap heparin column under reducing and nonreducing conditions, respectively (Table 3). In the presence of dithiothreitol, the elution properties of variants with a pair of cysteine residues were comparable to those observed for wt-rHCII. In the absence of the reducing agent, however, only 100–150 mM NaCl was required for elution of the cysteine-modified variants, suggesting that docking of the N terminus to a site close to helix D may interfere with heparin binding.

Enzyme-inhibiting properties of the open and closed forms of disulfide-engineered variants

wt-rHCII and variants were stably expressed in CHO cells, purified by immunoaffinity chromatography and assayed for their enzyme-inhibiting properties. Table 4 shows that in comparison with wt-rHCII, the α-chymotrypsin inhibition rate constant of the ΔC variant was little affected, indicating that replacement of the endogenous cysteine residues with serine had no marked effect on α-chymotrypsin inhibition. Covalent linkage of the N terminus to the serpin core of HCII resulted in a maximal 2.1-fold reduction of the

Table 3. Heparin affinity chromatography of heparin cofactor II (HCII) variants. The values shown in the columns indicate the NaCl concentrations at which each variant (peak fraction) eluted from HiTrap heparin-Sepharose. wt-rHCII, wild-type recombinant heparin cofactor II.

	Concentration of NaCl (mM)	
HCII variant	Dithiothreitol (3 mM)	Without dithiothreitol
wt-rHCII	200–250	200–250
ΔC/P52C/F195C	200–250	100–150
ΔC/G54C/F195C	200–250	100–150
ΔC/S68C/F195C	200–250	100–150

Table 4. Inhibition of α-chymotrypsin by recombinant heparin cofactor II (HCII) variants. The data shown are the rate constants for α-chymotrypsin inhibition. The values represent the mean ± SE of six to eight separate determinations. Assays were performed as described in the Materials and methods. wt-rHCII, wild-type recombinant heparin cofactor II.

HCII variant	$k_2 \cdot 10^5$ ($M^{-1} \cdot min^{-1}$)
wt-rHCII	2.5 ± 0.7
ΔC	1.5 ± 0.5
ΔC/F195C	1.2 ± 0.2
ΔC/P52C/F195C	1.2 ± 0.2
ΔC/G54C/F195C	1.4 ± 0.2
ΔC/S68C/F195C	1.2 ± 0.3

k_2 values, regardless of whether position 52, 54 or 68 was disulfide bonded to position 195, indicating that the tethered N terminus does not affect the interaction between the RSL region and α-chymotrypsin to a major extent.

Rates for α-thrombin inhibition were determined both in the absence and in the presence of GAGs. In the absence of GAGs, the k_2 values for α-thrombin inhibition of variants ΔC and ΔC/F195C were similar to those observed for wt-rHCII (Table 5), and treatment with dithiothreitol/iodoacetamide had no effect on this reaction. In contrast, mutants with an internal disulfide bridge behaved quite differently. In their reduced/alkylated forms, variants ΔC/P52C/F195C, ΔC/G54C/F195C, and ΔC/S68C/F195C displayed α-thrombin inhibition rates that were comparable with those of variants ΔC or ΔC/F195C. However, the unreduced forms of variants containing an internal disulfide depicted a very low α-thrombin inhibitory activity.

In the presence of 10 $U \cdot mL^{-1}$ heparin (Table 5), the reduced and iodoacetamide-treated forms of all variants inhibited α-thrombin very rapidly at a rate similar to that of wt-rHCII, or decreased maximally eightfold. Compared to wt-rHCII, the mutants depicted a more substrate-like behaviour, as indicated by the higher SI values (wt-rHCII, SI = 2.3; ΔC, ΔC/F195C, SI = 3.3; ΔC/P52C/F195C, ΔC/G54C/F195C, SI = 3.9; ΔC/S68C/F195C, SI = 5.0). The amount of heparin needed by these variants to achieve maximal α-thrombin inhibitory activity was not significantly changed compared to wt-rHCII (Fig. 4). In contrast, the unreduced forms of all variants with a tethered N terminus depicted k_2 values that were decreased at least 2670-fold compared to wt-rHCII or mutants without an internal disulfide. A low α-thrombin inhibitory activity was detected in the presence of heparin with the unreduced forms of all mutants containing a cysteine pair; however, because contamination with traces of the open conformation cannot be excluded, the significance of this observation is not clear.

Covalent docking of the N terminus to the serpin scaffold was accompanied by a strong decrease of the rate constants for α-thrombin inhibition also in the presence of dermatan sulfate (Table 5). However, compared to heparin, there was a stronger effect of the ΔC configuration on the dermatan sulfate-accelerated reaction, as all reduced mutants devoid of the genuine cysteine residues depicted k_2 values that – dependent on the type of variant – were lowered by ≈ eight- to 47-fold (at 100 $\mu g \cdot mL^{-1}$ dermatan sulfate) compared to the wt-rHCII control. In addition, slightly higher concentrations of dermatan sulfate were required for maximal α-thrombin inhibitory activity of mutants with the ΔC configuration (Fig. 4). The SI values obtained for α-thrombin inhibition in the presence of dermatan sulfate mimicked the results found with heparin (wt-rHCII, SI = 2.0; ΔC, SI = 2.7; ΔC/F195C, SI = 3.0; ΔC/P52C/F195C, SI = 3.1; ΔC/G54C/F195C, SI = 3.6; ΔC/S68C/F195C, SI = 5.0).

Substitution of the original amino acids at positions 52 and 54 by cysteine is associated with less severe effects concerning GAG-mediated α-thrombin inhibition after dithiothreitol/iodoacetamide treatment than the S68C mutation. This may reflect the fact that chemical modification of C68 by iodoacetamide creates a bulky side-chain that might interfere with the interaction between the inhibitor's N-terminal domain and exosite I of the target enzyme [12].

Discussion

Cysteine cross-linking is an established tool used to identify intramolecular contact sites in polypeptides and for studying mechanistic aspects of proteins [25,33–36]. Here we have engineered disulfide bridges to gain information on the location of the acidic N terminus of HCII in solution. In addition, we have investigated the consequences of reversibly locking this unique domain to the inhibitor's scaffold by measuring the enzyme-inhibitory properties of the mutants. The results demonstrate that the N terminus may interact with the loop region connecting helix D, the primary GAG-binding area of HCII, and strand s2A. As disulfide bridge formation is reformable *in vitro*, trapping of the N terminus by Cys195 is not caused by aberrant folding during synthesis. This conclusion is corroborated by the finding that locked HCII variants inhibit α-chymotrypsin at rates comparable to wt-rHCII, indicating that the mutants in their disulfide-bonded form depict a conformation that enables normal interaction with an important target enzyme of HCII.

Surprisingly, three different N-terminal sites may be tethered in a reformable manner to the exposed loop between strand s2A and helix D. Thus, there seems to be no strongly preferred solution conformation of the inhibitor's N-terminal extension, compatible with a permissive mode of

Table 5. Inhibition of α-thrombin in the presence and absence of glycosaminoglycans (GAGs). The data shown are the rate constants for α-thrombin inhibition. The values represent the mean ± SE of eight to 16 separate determinations. Assays were performed as described in the Materials and methods. HCII, heparin cofactor II; wt-rHCII, wild-type recombinant heparin cofactor II. Association constant, k_2 ($M^{-1}\cdot min^{-1}$), applies to all columns.

	No GAGs		Heparin (10 $U\cdot mL^{-1}$)		Dermatan sulfate (100 $\mu g\cdot mL^{-1}$)	
HCII variant	Unreduced	Reduced[a]	Unreduced	Reduced[a]	Unreduced	Reduced[a]
wt-rHCII	$2.9 \pm 0.5 \times 10^4$	$2.9 \pm 0.6 \times 10^4$	$9.2 \pm 0.5 \times 10^7$	$9.1 \pm 0.9 \times 10^7$	$9.3 \pm 0.7 \times 10^7$	$9.3 \pm 0.8 \times 10^7$
ΔC	$1.5 \pm 0.1 \times 10^4$	$1.6 \pm 0.3 \times 10^4$	$7.2 \pm 0.6 \times 10^7$	$7.0 \pm 0.3 \times 10^7$	$1.0 \pm 0.1 \times 10^7$	$8.7 \pm 0.9 \times 10^6$
ΔC/F195C	$1.9 \pm 0.4 \times 10^4$	$2.1 \pm 0.4 \times 10^4$	$7.3 \pm 0.7 \times 10^7$	$7.6 \pm 0.3 \times 10^7$	$1.0 \pm 0.1 \times 10^7$	$1.2 \pm 0.1 \times 10^7$
ΔC/P52C/F195C	$< 0.4 \times 10^4$	$2.1 \pm 0.3 \times 10^4$	$2.7 \pm 0.5 \times 10^4$	$7.3 \pm 0.4 \times 10^7$	$2.6 \pm 0.4 \times 10^4$	$1.0 \pm 0.2 \times 10^7$
ΔC/G54C/F195C	$< 0.4 \times 10^4$	$2.0 \pm 0.2 \times 10^4$	$2.3 \pm 0.5 \times 10^4$	$8.5 \pm 0.7 \times 10^7$	$2.1 \pm 0.6 \times 10^4$	$7.2 \pm 0.6 \times 10^6$
ΔC/S68C/F195C	$< 0.4 \times 10^4$	$1.1 \pm 0.3 \times 10^4$	$1.9 \pm 0.6 \times 10^4$	$1.2 \pm 0.1 \times 10^7$	$1.7 \pm 0.8 \times 10^4$	$2.0 \pm 0.1 \times 10^6$

[a] Including iodoacetamide treatment.

intramolecular interaction between the N terminus and the serpin body of HCII. It remains to be determined whether the contact sites identified here represent a selection of a larger set of intramolecular interactions. Protein domains with a large net charge have been found to be flexible with low levels of secondary structure [37,38]. The sequence encompassing positions 49–75 in the N terminus of HCII contains 15 acidic amino acids, including two sulfated tyrosine residues [21,39] that have been proposed to contact amino acids in the GAG-binding helix D, with the consequence that GAG binding is hindered [10,40]. In accordance with this suggestion, variants with an internal disulfide were eluted under 'low salt' conditions from a HiTrap heparin column, while in the presence of dithiothreitol, the 'high salt' conditions characteristic for wt-rHCII were required for desorption. Owing to the suggested flexibility of the N terminus, these intramolecular contacts might be variable and could include other areas in the HCII molecule. The existence of an equilibrium of different HCII conformers in solution has recently been proposed [11,41].

The effects of the covalent intramolecular linkage of the inhibitor's N-terminal domain were examined by measuring the inhibition rate constants for two target enzymes of HCII, α-chymotrypsin and α-thrombin. Intramolecular disulfide bond formation had little effect on α-chymotrypsin inhibition. Internally disulfide-bridged variants depicted similar second-order rate constants for this reaction as control variants containing no (ΔC) or only one (ΔC/F195C) cysteine residue. Compared with wt-rHCII, the k_2 values were only slightly affected. These observations confirm that the disulfide bond-containing variants are not aberrantly folded and that their RSL seems to be accessible for α-chymotrypsin in a normal manner. These data are consistent with previous findings demonstrating that the N terminus has a very limited role for α-chymotrypsin inhibition [10,11].

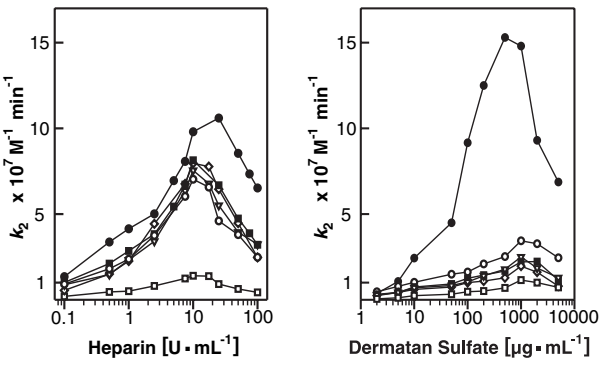

Fig. 4. Inhibition of α-thrombin by heparin cofactor II (HCII) variants in the presence of various concentrations of glycosaminoglycans (GAGs). Kinetic assays were performed, as described in the Materials and methods, with 5 nM α-thrombin and 50 nM of the inhibitor variants. The panels show the data for wt-rHCII (●), ΔC (○), ΔC/F195C (■), ΔC/P52C/F195C (◇), ΔC/G54C/F195C (▽) and ΔC/S68C/F195C (□) in the reduced state. The second-order rate constants represent an average of the values from at least three independent reactions.

The effects of intramolecular cross-linking on the interaction with α-thrombin, however, are different. A key conclusion from the data shown in Table 5 is that linkage of the N-terminal extension to the HCII body results in a drastic decrease of GAG-mediated α-thrombin inhibitory activity that is regained after cleavage of the disulfide bond, suggesting that liberation of the N terminus from intramolecular interactions is an essential aspect of GAG-mediated α-thrombin inhibition. Consistent with this data, only trace amounts of SDS stable inhibitor–α-thrombin complexes were detected with mutants having a docked N terminus, irrespective of the presence of GAGs. Liberation of this domain by reduction, however, resulted in the appearance of undegraded complexes, as indicated by Western blot analysis (data not shown).

Docking of the N terminus to the HCII body, or deletion of the 74 N-terminal amino acids [10], is associated with similar consequences with respect to GAG-mediated inactivation of α-thrombin and inhibition of α-chymotrypsin. α-Thrombin inactivation in the presence of GAGs is strongly impaired by either kind of mutation, while there is a only a modest decrease of the α-chymotrypsin inhibition rates [10,17,42] (also shown in this work). With respect to α-thrombin inhibition in the absence of GAGs, slightly increased [17,42] or decreased [10] second-order rate constants have been reported for the deletion mutant. In contrast, strongly lowered k_2 values were observed for the variants with a tethered N terminus. This may reflect interference of the attached N terminus with the expulsion of the distal region of the RSL, which may be partially incorporated into β-sheet A [12] and which is located close

to the loop connecting strand 2A and helix D (Fig. 1). Alternatively, reversible dissociation of the N terminus from intramolecular interactions could contribute to α-thrombin inhibition also in the absence of GAGs.

The biochemical properties of the variants analysed in this study unravel some differences between the heparin and the dermatan sulfate-mediated HCII–α-thrombin interaction. Using a sulfhydryl derivatization procedure it was demonstrated [31] that the endogenous cysteine residues do not have a significant role in heparin-mediated α-thrombin inhibition by HCII. In accordance with these findings, substitution of the three endogenous cysteine residues by serine (variant ΔC) did not modulate heparin-mediated α-thrombin inhibition to a major extent (Table 5), and no major influence was found on α-chymotrypsin inhibition. In contrast, variant ΔC and all other variants in which the endogenous cysteine residues were replaced by serine displayed lowered k_2 values for dermatan sulfate-mediated α-thrombin inhibition, suggesting that the effect is GAG selective. In addition, the dermatan sulfate concentration required for maximal α-thrombin inhibitory activity was shifted to slightly higher values with these mutants. These features may point to mechanistic differences between the heparin- and dermatan sulfate-catalyzed inhibitor/enzyme reactions and require further investigation.

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