Interaction of Heparin Cofactor II with Neutrophil Elastase and Cathepsin G*

Charlotte W. Pratt, Rebecca B. Tobin, and Frank C. Church

From the Departments of Pathology and Medicine, The Center for Thrombosis and Hemostasis, The University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7035

We investigated the interaction of the human plasma proteinase inhibitor heparin cofactor II (HC) with human neutrophil elastase and cathepsin G in order to examine 1) proteinase inhibition by HC, 2) inactivation of HC, and 3) the effect of glycosaminoglycans on inhibition and inactivation. We found that HC inhibited cathepsin G, but not elastase, with a rate constant of $6.0 \times 10^6 \text{M}^{-1}\text{min}^{-1}$. Inhibition was stable, with a dissociation rate constant of $1.0 \times 10^{-6} \text{min}^{-1}$. Heparin and dermatan sulfate diminished inhibition slightly. Both neutrophil elastase and cathepsin G at catalytic concentrations destroyed the thrombin inhibition activity of HC. Inactivation was accompanied by a dramatic increase in heat stability, as occurs with other serine proteinase inhibitors. Proteolysis of HC ($M_r$ 66,000) produced a species ($M_r$ 58,000) that retained thrombin inhibition activity, and an inactive species of $M_r$ 48,000. Amino acid sequence analysis led to the conclusion that both neutrophil elastase and cathepsin G cleave HC at Ile$^{66}$, which does not affect HC activity, and at Val$^{493}$, near the reactive site Leu$^{444}$, which inactivates HC. Since cathepsin G is inhibited by HC and also inactivates HC, we conclude that cathepsin G participates in both reactions simultaneously so that small amounts of cathepsin G can inactive a molar excess of HC. High concentrations of heparin and dermatan sulfate accelerated inactivation of HC by neutrophil proteinases, with heparin having a greater effect. Heparin and dermatan sulfate appeared to alter the pattern, and not just the rate, of proteolysis of HC. We conclude that while HC is an effective inhibitor of cathepsin G, it can be proteolyzed by neutrophil proteinases to generate first an active inhibitor and then an inactive molecule. This two-step mechanism might be important in the generation of chemotactic activity from the amino-terminal region of HC.

During the acute inflammatory response, neutrophils respond to a variety of endogenous and exogenous stimuli by migrating to sites of injury and releasing substances that damage pathogens as well as host tissue (1). Among the hydrolytic enzymes released are serine proteinases. These enzymes are believed to be regulated in vitro by members of the serine proteinase inhibitor (serpin) family of proteins. Interestingly, neutrophils and their released products can also inactivate many serpins not only by oxidizing a critical methionine residue in $\alpha_1$-proteinase inhibitor (2, 3) but also by cleaving peptide bonds in an exposed loop near the reactive sites of several other serpins (5-8). The balance between proteinase activity and proteinase inhibition is critical; disruption of the balance might contribute to disease states such as emphysema and rheumatoid arthritis.

We are interested in the properties of the plasma proteinase inhibitor heparin cofactor II (HC), which is a member of the serpin family. HC, like its homologue antithrombin III, inhibits thrombin in a reaction whose rate is dramatically increased by heparin and other glycosaminoglycans (9). This property suggests a potential role for thrombin inhibition by HC in vivo at sites rich in glycosaminoglycans, such as vessel walls and exposed basement membranes. The physiological importance of HC might also depend on the production of leukocyte chemotactants from the HC protein, as we recently reported (10).

We investigated the interaction of HC with two major neutrophil proteinases, elastase and cathepsin G. Because HC is a potent inhibitor of chymotrypsin (11), it might inhibit cathepsin G, which has similar hydrolytic specificity (12). On the other hand, neutrophil elastase, which inactivates the related serpins antithrombin III, $\alpha_2$-antiplasmin, $\alpha_1$-antichymotrypsin, and C1 inhibitor (4, 5, 8), might also inactivate HC. Sie et al. (13) have shown that neutrophil elastase degrades and inactivates HC, but the degrading activity was not identified. It is known that neutrophil elastase proteolytically inactivates antithrombin III in a reaction that is accelerated by heparin (8, 14); however, the effect of glycosaminoglycans on HC inactivation by purified neutrophil proteinases is not known.

In this study, we found that HC inhibits cathepsin G, but not elastase, and that both cathepsin G and elastase inactivate HC by a proteolytic reaction that is accelerated by heparin and dermatan sulfate. Additionally, we found that the initial product of the reaction between HC and neutrophil proteinases is a degraded form of HC that retains its inhibitory properties. A preliminary report has appeared in abstract form (15).

Experimental Procedures

Materials—HC was purified from human plasma as described (16). HC concentrations were determined using an extinction coefficient of 0.59 ml mg$^{-1}$ cm$^{-1}$ at 280 nm (17). Human neutrophil elastase and cathepsin G purchased from Elastin Products (Pacific, MO) were assumed to be 100% active and were stored in 0.2 M sodium acetate.

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† To whom correspondence should be addressed: Div. of Hematology, Campus Box 7035, 416 Burnett-Womack Building, The University of North Carolina, Chapel Hill, NC 27599-7035.
pH 5.0, at −20 °C. Human α-thrombin was purified as described (18) and active site-titrated with p-nitrophenyl-p′-guanidinobenzoate (Sigma) (19). Heparin was provided by Diosynth (Oss, Netherlands). Dermatan sulfate from Calbiochem was treated with nitrous acid to remove heparin contaminants (20). All experiments were performed in 20 mM HEPES, 150 mM sodium chloride, 0.1% polyethylene glycol 8000 (Triton X-100, pH 7.4, at 25 °C).

**Protease Inhibition—**Cathepsin G activity was assayed with 0.2 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma); neutrophil elastase activity was assayed with 0.2 mM methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma). For cathepsin G, the inhibition rate constant (k−1) was determined under second-order conditions using 100 nM HC, 5 nM thrombin, 2 mg/ml bovine serum albumin, and 10 pg/ml dermatan sulfate for various times from 5 to 30 s, 700 µl of 0.3 mM substrate was added. Hydrolysis was terminated after 30 min with acetic acid. Absorbance at 405 nm was linearly related to cathepsin G concentration. The equation used to calculate k−1 was the standard second-order rate equation: 1/[P]t = 1/[P]0 + k−1t, where [P]t and [P]0 are proteinase concentrations at a time t or at t = 0, respectively (21). Dissociation of inhibition was measured with the same protein concentrations in 1 ml. Aliquots of 100 µl were removed at intervals of 0.5–5 h and added to 700 µl of substrate. The reaction was terminated after 30 min. The dissociation rate constant (k−1) was calculated using the standard first-order rate equation: ln[P]t/[P]0 = k−1t (21). Kinetic measurements were also performed in the presence of 50 pg/ml heparin or 100 pg/ml dermatan sulfate. Experiments were performed from 2 to 5 times and the results averaged.

Additional experiments with cathepsin G inhibition involved 500 nM HC and 500 nM cathepsin G in 400 µl. Aliquots of 70 µl were removed at intervals of 10–50 s and added to 100 µl of substrate. The reaction was stopped after 15 min by the addition of 25 µl of 50% acetic acid, and the absorbance at 405 nm was measured in a microplate reader. These experiments were performed 6 times.

**HC Activity Assays—**Thrombin inhibition activity of HC treated with elastase or cathepsin G was measured by incubating 2 µM HC with 20 nM elastase or 50 nM cathepsin G in 400 µl. At intervals, 70 µl aliquots were removed and added to 30 µl of S-2302 (Kabi) to produce band III; HC was incubated with 50 nM cathepsin G for 50 min (band II) and for 4 h (band III). PMSF was added (0.5 mM) to stop reaction after 50 s by the addition of 30 µl of 50% acetic acid. The 5-min incubation allowed complete reaction between thrombin and HC so that the activity of thrombin was linearly related to the concentration of active HC. The proportion of HC inactivated by cathepsin G (expressed as relative to the uninhibited control) ([HC]sample/[HC]control) was calculated using the following equation: [HC]sample/[HC]control = (1 − [P]sample/[P]0 − [P]uninhibited), where [P] is thrombin activity relative to thrombin in the absence of HC and (1 − a) is inhibition activity. These experiments were performed 6 times.

**Electrophoresis—**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% slab gels (22) with Rainbow molecular weight markers (Amersham Corp.). The apparent relative molecular weights of HC and its derivatives were higher than expected; calculated molecular weights were normalized to a molecular weight of 66,000 for native HC (23). The possibility of additional proteolysis after addition of SDS was ruled out by the observation that phenylmethylsulphonyl fluoride (PMSF) added prior to SDS did not alter the results.

**Heat Stability Assays—**HC (7.6 µM or 0.5 mg/ml) was incubated with 76 nM elastase for 1 h or with 76 nM cathepsin G for 3 h (conditions under which thrombin inhibition activity was completely lost) and then treated with 0.1 mM PMSF. A 500-µl sample was placed in a 50 °C water bath; 50-µl samples were removed at intervals of 5 min and centrifuged, and protein remaining in solution was assayed using a Coomassie Blue G-250 dye-binding assay (Pierce Chemical Co.). The results of 2–3 experiments were averaged.

**Preparation of Bands II and III—**HC (2 µM) was incubated with 20 nM elastase for 12 min to produce band II and for 40 min to produce band III; HC was incubated with 50 nM cathepsin G for 50 min (band II) and 4 h (band III). PMSF was added (0.5 mM) to stop reaction. Following dialysis, samples were analyzed by SDS-PAGE, and the Pierce dye-binding assay was used to measure protein content (115–130 mg/ml) with HC as a standard. The molarity of the HC fragments was calculated from the protein content and apparent molecular weight of 100 µg/ml PMSF. AsSays for HC activity used the following conditions: for thrombin inhibition assays: 500 nM HC (or HC fragment), 5 nM thrombin, 2 mg/ml bovine serum albumin, reaction time 30 min; for heparin cofactor assays: 100 nM HC, 5 nM thrombin, 2 mg/ml bovine serum albumin, 10 µg/ml heparin, reaction time 10 s; for dermatan sulfate cofactor assays: 100 nM HC, 5 nM thrombin, 2 mg/ml bovine serum albumin, 10 µg/ml dermatan sulfate, reaction time 1 min. Active HC concentrations were determined as used under "HC activity assays." The entire experiment was performed twice, and HC activity assays were performed 3 times.

**Protein Sequencing—**Samples contained 2 µM HC digested with 20 nM elastase (15 min) or 50 nM cathepsin G (2 h) and were treated with PMSF and dialyzed against 5% acetic acid before sequence analysis. Digests were analyzed by the Protein Chemistry Laboratory of the University of North Carolina at Chapel Hill on an Applied Biosystems 475A protein sequencer.

**RESULTS**

**Protease Inhibition—**Inhibition of amidolytic activity of neutrophil elastase and cathepsin G by HC was investigated using chromogenic substrates for each protease. Minimal inhibition of elastase was detected (50% inhibition of 25 nM elastase by 7 µM HC) and was not further characterized. In contrast, HC inhibited cathepsin G with a second-order rate constant of 6.0 × 10^−6 M^−1 min^−1 (Table I). Assuming a plasma concentration of 1 µM for HC, this translates to a half-time for cathepsin G inhibition of 10 s (t_1/2 = 1/k+1[HC]), which may have relevance in vivo (24). The first-order dissociation rate constant for inhibition was also determined: 1.0 × 10^−3 min^−1 (equivalent to a half-life of about 11 h). Heparin and dermatan sulfate decreased the inhibition rate constant slightly and increased the dissociation rate constant, indicating less stable inhibition (Table I). A bimolecular complex containing HC and cathepsin G was not visible by SDS-PAGE. However, during gel filtration of mixtures of HC and cathepsin G on Sepharcl S-200, cathepsin G activity eluted with the HC peak (not shown).

**Inactivation of HC—**Both neutrophil elastase and cathepsin G at catalytic concentrations inactivated HC, as measured by loss of thrombin inhibition activity (Fig. 1). Neutrophil elastase...

**Table I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>k+a</th>
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<tr>
<td>None</td>
<td>46</td>
<td>1.0</td>
</tr>
<tr>
<td>Heparin (50 µg/ml)</td>
<td>4.2</td>
<td>6.4</td>
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<tr>
<td>Dermatan sulfate (50 µg/ml)</td>
<td>4.7</td>
<td>3.1</td>
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FK;.

1. Inactivation of heparin cofactor II by neutrophil proteinases. HC (2 μM) was incubated with 20 nM neutrophil elastase (●), or 50 nM cathepsin G (▲), or without proteinase (○), and then assayed for thrombin inhibition activity. Data are expressed as HC activity relative to the control sample at zero time.

2. Heat stability of heparin cofactor II. HC (○), HC inactivated by neutrophil elastase (●), and HC inactivated by cathepsin G (▲) were incubated at 58 °C and then assayed for protein remaining in solution. No correction was made for evaporation.

3. Activity of bands II and III derived from heparin cofactor II. HC was incubated with neutrophil elastase and cathepsin G to produce bands II and III as described under "Experimental Procedures." Samples were analyzed by SDS-PAGE (A) and assayed for thrombin inhibition activity (B). Lane a, HC control. Lane b, HC digested with elastase (band II). Lane c, HC digested with elastase (band III). Lane d, HC digested with cathepsin G (band II). Lane e, HC digested with cathepsin G (band III). The antithrombin (∆), heparin cofactor (□) and dermatan sulfate (■) cofactor activities of preparations b–e are expressed relative to the HC control (sample a).

Heparin Cofactor II and Neutrophil Elastase and Cathepsin G

Amino acid sequence analysis was performed on HC-proteinase digests to identify sites of proteolysis by neutrophil elastase or cathepsin G.
elastase and cathepsin G. In analyses of mixtures of bands II and III, multiple residues were obtained at each cycle. In both neutrophil elastase and cathepsin G digests the amino-terminal sequence, the sequence beginning with Phe's, and the sequence beginning with Gly's, were identified. Neither proteinase cleaved the reactive site bond: Leu-Ser (26). No other sequences were unambiguously identified in cathepsin G digests, but elastase digestion also produced a new amino-terminal sequence beginning with Asp (21) and the terminal sequence beginning with Asp (29) (Table II).

Relationship between Cathepsin G Inhibition and HC Inactivation—In order to directly compare HC inactivation with cathepsin G inhibition, both reactions were followed under identical conditions of time and concentration. As shown in Fig. 4A, both inhibition and inactivation occurred in the same interval. The calculated second-order rate constants were 6.2 × 10^6 M^{-1} min^{-1} for cathepsin G inhibition and 1.6 × 10^6 M^{-1} min^{-1} for HC inactivation. These rate constants are probably overestimations since the reactions are simultaneous: some apparent inability of HC to inhibit thrombin might be the result of cathepsin G inhibition. This was also demonstrated by intercepts of greater than unity in the replot (Fig. 4B). When the data were normalized to give intercepts of unity, the rate constants became 3.6 × 10^6 M^{-1} min^{-1} for cathepsin G inhibition and 1.4 × 10^6 M^{-1} min^{-1} for HC inactivation. Under the same reaction conditions, the dissociation rate constant for cathepsin G inhibition was 3.5 × 10^{-3} min^{-1} (half-life of 3.3 h; not shown), thus demonstrating that inactivation of HC by cathepsin G did not occur following dissociation of cathepsin G. The stability of cathepsin G inhibition by HC was further demonstrated by the inability of excess thrombin to promote cathepsin G dissociation from HC (not shown), thus demonstrating that inactivation of HC by cathepsin G is irreversible.

Effect of Heparin and Dermatan Sulfate on HC Inactivation—Because glycosaminoglycans such as heparin and dermatan sulfate alter the rate of thrombin inhibition by HC,
The inactivation of HC at high concentrations of heparin and dermatan sulfate resulted in altered patterns of degradation of HC protein as visualized by SDS-PAGE (Fig. 6). When heparin was included, a band similar to band III predominated, and HC activity was greatly reduced. When dermatan sulfate was included, HC activity was reduced, although proteolysis appeared to be diminished relative to controls in the absence of glycosaminoglycan. A band similar to band II was apparent in samples containing dermatan sulfate, but this raises the possibility that this species was not identical to band II, which retains inhibitory activity. It is possible that glycosaminoglycans alter the susceptibility of HC to proteolysis such that inactivation at the reactive site can occur without extensive proteolysis in the amino-terminal region of the HC molecule.

**DISCUSSION**

This study was undertaken to determine whether HC inhibits the hydrolytic activity of two major neutrophil proteinases or is inactivated by them. Our results demonstrate that HC inhibits cathepsin G under physiological conditions, but that both cathepsin G and elastase can proteolyze and destroy the thrombin inhibition activity of HC. The inhibition of cathepsin G by HC might be important in vivo as a supplement to α1-antichymotrypsin inhibition of cathepsin G (27). The less stable inhibition obtained in the presence of glycosaminoglycans most likely reflects binding of the proteinase to the polyanion, also noted by others (28). The absence of a covalent SDS-stable HC-cathepsin G complex is similar to results with HC-chymotrypsin (11) and α1-proteinase inhibitor-trypsin (29). However, the failure to detect the sequence beginning with Ser445 suggests that cathepsin G does not actually hydrolyze the Leu444-Ser bond. Our inhibition measurements differ significantly from those of a previous study which reported an inhibition rate constant of only 1.4 × 104 M⁻¹ min⁻¹, possibly due to different reaction temperatures (30). The mechanism of cathepsin G inhibition is discussed further below.

The inactivation of HC by neutrophil elastase or cathepsin G was accompanied by increased heat stability, which is consistent with a conformational change caused by proteolytic cleavage of the reactive site peptide loop of HC. This is the first report of the heat stability phenomenon in HC, which has been well documented for other members of the serpin family (25, 31). Proteolysis of the reactive site peptide loop renders other serpins inactive as proteinase inhibitors (31). Sequence data provided strong evidence that the inactivation of HC by neutrophil elastase and cathepsin G was accomplished by proteolysis of the reactive site loop at Val₄⁴⁴-Gly (the P₄-P₃ bond in the nomenclature of Schechter and Berger (32)).

Both neutrophil elastase and cathepsin G also hydrolyze HC near the amino terminus, at the Ile₄⁶⁶-Phe bond, as shown by sequence analysis. We conclude that this cleavage produces band II in SDS-PAGE, which retains thrombin inhibition activity. This is not unexpected since the reactive site of HC is located near the carboxyl terminus of HC (26). We have previously identified a degraded form of HC (with an amino terminus corresponding to Asn₄⁵⁶) that contains an intact reactive site (16). The results of HC activity assays and SDS-PAGE analysis indicate that the Ile₄⁶⁶-Phe bond is cleaved before the Val₄⁴⁴-Gly bond. Neutrophil elastase and cathepsin G generate the same proteolytic fragments from HC. HC differs from antithrombin III and α₂-antiplasmin in that proteolysis of HC by neutrophil proteinases initially generates a degraded form of the inhibitor that retains activity. Only
C1 inhibitor appears to share this feature (6).

The effect of heparin and dermatan sulfate on the inactivation of HC by neutrophil proteinases is not correlated with the effect of the glycosaminoglycan on proteinase activity (as assessed with small chromogenic substrates) but is consistent with glycosaminoglycan binding to HC to alter its susceptibility to proteolysis. The accelerated inactivation of HC in the presence of high concentrations of glycosaminoglycans might involve a ternary complex consisting of glycosaminoglycan, inhibitor, and proteinase (similar to the ternary complex formed during thrombin inhibition by HIC and glycosaminoglycan (33)). The greater effect of heparin compared to dermatan sulfate parallels the tighter binding of heparin to HC (9), but might also reflect a different binding mechanism. Our data show that the heparin effect at least is markedly concentration-dependent. The protective effect of heparin at low concentrations is at present unexplained, although it is possible that this reflects a heparin-induced decrease in proteinase activity, which was observed with elastase but which was not detected when cathepsin G was assayed with a synthetic peptide substrate. Sie et al. (13) reported that 1 μg/ml heparin protected HC from inactivation by neutrophil lysates, while 10 μg/ml dermatan sulfate had no effect; higher glycosaminoglycan concentrations were not tested. Our results extend the observations of Sie et al. (13) and demonstrate that HC shares with antithrombin III the property of heparin-accelerated inactivation by neutrophil elastase. The results of SDS-PAGE indicate that glycosaminoglycans do not simply increase the rate of conversion of HC to band II and then band III, but probably promote proteolysis near the reactive site, thus rendering HC inactive with respect to thrombin inhibition.

To resolve the paradox in which cathepsin G is inhibited by HC but also inactivates the inhibitor, there are two possibilities. One is that the interaction of cathepsin G with the HC-cathepsin G complex has a half-time of hours. Neither thrombin from reacting at Leu444-Ser. However, this does not explain how small amounts of cathepsin G can inactivate a large molar excess of HC since the apparent dissociation of the HC-cathepsin G complex has a half-time of hours. Nor does it explain why the rate constants for cathepsin G inhibition and HC inactivation are different. The second possibility is that cathepsin G participates in the inhibition and inactivation reactions simultaneously and independently. Inhibition of cathepsin G probably occurs at the reactive site of HC (as with the similar proteinase chymotrypsin (11)), although the typical serpin complex, which is SDS-stable (24) is not detected, and the Leu444-Ser bond is not completely hydrolyzed. During the course of the cathepsin G inhibition reaction, cathepsin G also reacts with HC first near the amino terminus to cleave the Ile65-Phe bond without affecting proteinase inhibition activity, and then near the carboxyl terminus to further destroy HC inhibition activity by cleaving the Val199-Gly bond. In the presence of a large molar excess of HC, not all cathepsin G would be inhibited; some would be available to participate in HC inactivation. The second possibility also explains how cathepsin G can remain inhibited (at Leu444) after complete conversion of HC to band III (cleavage at Val199).

We have recently found that neutrophil elastase and cathepsin G generate leukocyte chemotaxins from the amino-terminal portion of HC (10). The results of this paper demonstrate that proteolysis at Ile65-Phe occurs without diminishing proteinase inhibition activity near the carboxyl terminus. Although it is not known whether heparin or dermatan sulfate influence the production of chemotactic activity, glycosaminoglycans might play a role in defining the susceptibility of HC to proteolysis with or without inactivation of HC.

REFERENCES


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