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 \(6.0 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}\). Inhibition was stable, with a dissociation rate constant of \(1.0 \times 10^{-3} \text{ min}^{-1}\). 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\(^{48}\), which does not affect HC activity, and at Val\(^{148}\), near the reactive site Leu\(^{444}\), which inactivates HC. Since cathepsin G is inhibited by HC and also inactivates HC, we conclude that the reaction is accelerated by heparin and other glycosaminoglycans (9). This property suggests a potential role for 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 chemoattractants 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, \(\alpha_1\)-antiplasmin, \(\alpha_1\)-antichymotrypsin, and Cl inhibitor (4, 5, 8), might also inactivate HC. Sie et al. (13) have shown that neutrophil extracts degrade and inactivate 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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Heparin Cofactor II and Neutrophil Elastase and Cathepsin G

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, 0.03% Triton X-100, pH 7.4, at 25 °C.

**Proteinase 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 methoxy succinyl-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 active proteinase in the presence of 100 nM substrate at 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] = k·t + 1/[P]0, where [P], 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]/[P]0 = k−1·t (21). Kinetic measurements were also performed with 50 µg/ml heparin or 100 µg/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 a thrombin inhibition assay with final concentrations of 1.4 µM HC, 6 nM thrombin, and 2 mg/ml Polybrene (Aldrich). After 10 min, 650 µl of 0.3 mM tosyl-Gly-Pro-Arg-p-nitroanilide (Boehringer Mannheim) was added, and hydrolysis was stopped after 5 min by the addition of acetic acid. For some thrombin inhibition assays, reaction conditions were: 800 nM HC, 5 nM thrombin, 2 mg/ml bovine serum albumin (Sigma), 0.1 mg/ml Polybrene, reaction time 30 min. Experiments were performed at least 3 times and the results averaged. Control experiments demonstrated that elastase and cathepsin G present in the HC samples had no effect on thrombin activity or substrate hydrolysis. The active HC concentration in a sample was calculated from thrombin activity measurements. Since the pseudo-first-order rate equation for thrombin inhibition by excess HC in k−1 = -ln a/[HC], where a is thrombin activity relative to the uninhibited control, t is time, and k is the rate constant, then [HC] = -ln a/kt. To express inactivation of HC by elastase or cathepsin G (HCsample) relative to untreated HC (HCcontrol), k and t were treated as constants so that [HCsample]/[HCcontrol] = ln aHCsample/ln aHCcontrol. These calculations are based on the assumption that elastase and cathepsin G affect the concentration or activity of HC but do not affect the rate constant for thrombin inhibition. For digests in the presence of heparin or dermatan sulfate, HC was incubated with neutrophil proteinases for various times, and then 2 aliquots were removed and 1) added to the thrombin inhibition assay or 2) added to SDS for denaturation and SDS-PAGE. These experiments were performed 3 times.

Additional experiments with cathepsin G involved 500 nM HC treated with 500 nM cathepsin G in 400 µl. At intervals of 10–50 s, 70-µl aliquots were removed and added to a thrombin inhibition assay with 20 nM thrombin and 100 nM dermatan sulfate, 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; 500-µl samples were removed at 5-min intervals 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 proteolysis. Following dialysis, samples were analyzed by SDS-PAGE, and the Pierce dye-binding assay was used to measure protein content (115-130 µg/ml) with HC as a standard. The molarity of the HC fragments was calculated from the protein content and apparent molecular weight in SDS-PAGE. As 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 described 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 20 nM cathepsin G (2 h) and were treated with 0.5 mM PMSF and dialyzed against 100 mM NaOH at pH 11. Peptide sequences were determined using a PMSF dialysis against 100 mM NaOH at pH 11. Peptide sequences were determined using a Protein Chemistry Laboratory of the University of North Carolina at Chapel Hill on an Applied Biosystems 475A protein sequencer.

**RESULTS**

**Proteinase Inhibition**—Inhibition of amidolytic activity of neutrophil elastase and cathepsin G by HC was investigated using chromogenic substrates for each proteinase. 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 × 107 M−1 s−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 (t1/2 = 1/k[Hc]) (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 Sephacryl 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 was expressed relative to both HC concentration and the thrombin inhibition activity. These experiments were performed 6 times.

**Electrophoresis—** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% slab gels (22) without chemical reduction of samples, 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 phenylmethanesulfonyl fluoride (PMSF) added prior to SDS did not alter the results.

**TABLE I**

| Addition          | k1  | k−1 |
|-------------------|-----|-----|
|                   | 10−8 s−1 M−1 | 10−6 s−1 |
| None              | 6.0 | 6.4 |
| Heparin (50 µg/ml)| 4.2 | 6.4 |
| Dermatan sulfate (50 µg/ml)| 4.7 | 3.1 |
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.

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.

The two proteinases generated HC derivatives of similar mobility in SDS-PAGE. During the time course of HC inactivation, two major degradation products were observed; the apparent molecular weights of these were: 58,000 ("band II") and 48,000 ("band III"), compared to native HC (66,000) (Fig. 3A). Band II appeared first and was converted to band III over time. Extended incubation of HC (up to 53 h) with elastase did not produce additional degradation of band III; however, incubation with cathepsin G produced multiple lower molecular weight bands and material migrating with the dye front in SDS-PAGE (not shown). These results suggest that while the two enzymes generate HC fragments by proteolysis at or near the same sites initially, the ultimate products of proteolysis by neutrophil elastase or cathepsin G are different.

Preliminary observations suggested that HC digests containing band II retained some thrombin inhibition activity. This was further quantified by timed incubation of HC with neutrophil elastase and cathepsin G to prepare HC digests enriched in band II or 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).

Further evidence for the presence of an intact reactive site for thrombin inhibition in band II preparations was obtained by incubating these preparations with thrombin, and visualizing higher molecular weight complexes in SDS-PAGE (not shown).

Amino acid sequence analysis was performed on HC-proteinase digests to identify sites of proteolysis by neutrophil...
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 (26), and the sequence beginning with Gly (35) were identified. Neither proteinase cleaved the reactive site bond: Leu (44)-Ser (26). No other sequences were unambiguously identified in cathepsin G digests, but elastase digestion also produced a new amino-terminal sequence beginning with Phe (104). Multiple residues were obtained at each cycle. In both neutrophil elastase and cathepsin G digestion, the amino-terminal sequence beginning with Asp (21) and the sequence beginning with Tyr (15) were identified. The reactive site bond is Leu (44)-Ser (26).

### Table II

| Cycle | Aminoterminal sequences of heparin cofactor II digested with neutrophil elastase and cathepsin G |
|-------|------------------------------------------------------------------------------------------|
| 1     | Elastase                                                                                   |
|       | Gly (142)                                                                                  |
|       | Ser (28)                                                                                   |
|       | Lys (35)                                                                                   |
| 2     | Phe (129)                                                                                  |
|       | His (35)                                                                                   |
| 3     | Phe (104)                                                                                  |
|       | Met (32)                                                                                   |
| 4     | Phe (104)                                                                                  |
|       | Met (32)                                                                                   |
| 5     | Phe (104)                                                                                  |
|       | Met (32)                                                                                   |
| 6     | Phe (104)                                                                                  |
|       | Met (32)                                                                                   |
| 7     | Phe (104)                                                                                  |
|       | Met (32)                                                                                   |
| 8     | Phe (104)                                                                                  |
|       | Met (32)                                                                                   |

Amino-terminal sequences

Elastase

Gly (142)
Ser (28)
Lys (35)
Phe (129)
His (35)
Phe (104)
Met (32)
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and because heparin has been reported to accelerate the rate of inactivation of antithrombin III by elastase (8), the effect of glycosaminoglycans on the inactivation of HC was investigated. Following incubation of HC with neutrophil elastase or cathepsin G in the presence of varying amounts of heparin or dermatan sulfate (0.1 μg/ml to 3 mg/ml), thrombin inhibition was measured. Increasing heparin concentrations initially afforded slight protection of HC from inactivation by neutrophil proteinases, but above about 10 μg/ml, heparin accelerated HC inactivation (Fig. 5A). In contrast, dermatan sulfate at all concentrations caused only a slight increase in the rate of HC inactivation (Fig. 5B). The effect of glycosaminoglycans on HC inactivation was not correlated with the effect of glycosaminoglycans on the activity of each proteinase as assayed by small chromogenic substrates. For example, HC inactivation was increased at high concentrations of dermatan sulfate, where both elastase and cathepsin G activities were depressed (Fig. 5D). Furthermore, the concentration dependence of the heparin effect on HC inactivation was similar for neutrophil elastase and cathepsin G, even though the amidolytic activity of these enzymes responded differently to various heparin concentrations (Fig. 5C).

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 Ser45 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 × 10^4 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 Val450-Gly (the P1-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 Ile66-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 Asn165) that contains an intact reactive site (16). The results of HC activity assays and SDS-PAGE analysis indicate that the Ile66-Phe bond is cleaved before the Val450-Gly bond. Neutrophil elastase and cathepsin G generate the same proteolytic fragments from HC. HC differs from antithrombin III and α1-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 IIC 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 Val<sup>139</sup>-Gly bond results in stable inhibition and prevents thrombin from reacting at Leu<sup>444</sup>-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 Leu<sup>444</sup>-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 Ile<sup>46</sup>-Phe bond without affecting proteinase inhibition activity, and then near the carboxyl terminus to further destroy HC inhibition activity by cleaving the Val<sup>139</sup>-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 Leu<sup>444</sup>) after complete conversion of HC to band III (cleavage at Val<sup>139</sup>).

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 Ile<sup>46</sup>-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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**Footnote:**

2 F. C. Church, C. W. Pratt, and M. Hoffman, manuscript in preparation.