The Protease Specificity of Heparin Cofactor II

INHIBITION OF THROMBIN GENERATED DURING COAGULATION*

(Received for publication, September 24, 1984)

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125I-labeled heparin cofactor II (HCII) was mixed with plasma and coagulation was initiated by addition of CaCl2, phospholipids, and kaolin or tissue factor. In the presence of 67 μg/ml of dermatan sulfate, radioactivity was detected in a band which corresponded to the thrombin-HCII complex (Mw = 96,000) upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No other complexes were observed. The thrombin-HCII complex was undetectable when 5 units/ml of heparin was present or when prothrombin-deficient plasma was used. In experiments with purified proteases, HCII did not significantly inhibit coagulation factors VIIa, IXa, Xa, XIa, XIIa, and kallikrein, activated protein C, plasmin, urokinase, tissue plasminogen activator, leukocyte elastase, the γ-subunit of nerve growth factor, and the epidermal growth factor-binding protein. HCII inhibited leukocyte cathepsin G slowly, with a rate constant of 8 × 104 M⁻¹ min⁻¹ in the presence of dermatan sulfate. These results indicate that the protease specificity of HCII is more restricted than that of other plasma protease inhibitors and suggest that the anticoagulant effect of dermatan sulfate is due solely to inhibition of thrombin by HCII.

Heparin cofactor II (HCII) is a 65,600-dalton glycoprotein in human plasma which inhibits thrombin by forming a stable, equimolar complex with the protease (1–7). Heparin and dermatan sulfate bind to HCII and thereby increase the rate of inhibition of thrombin ~1000-fold (4, 5, 8). Heparin also stabilizes the inhibition of thrombin and other proteases by antithrombin III (ATIII) (9). In contrast, dermatan sulfate specifically catalyzes the thrombin-HCII reaction but has no appreciable effect on the activity of ATIII (5, 10). HCII is present in plasma at a concentration of ~1.2 μM.† At this concentration, thrombin, could theoretically be inhibited with a t½ approaching 50–100 ms in the presence of an optimal amount of heparin or dermatan sulfate.

Little information is available about the protease specificity of HCII. In previous studies, HCII did not inhibit coagulation factor Xa, plasmin, or trypsin (2, 4, 6, 7). In contrast, ATIII inhibits a broad range of proteolytic enzymes, including the coagulation factors thrombin, Xa, IXa, XIa, XIIa, and kallikrein, and the fibrinolytic enzyme plasmin (9, 11). We have now examined all of the proteases known to be involved in coagulation and fibrinolysis, as well as several other extracellular proteases, and have discovered that HCII is a relatively specific inhibitor of thrombin. Previously we reported that 125I-thrombin added to plasma containing dermatan sulfate becomes bound to HCII (5). We have now shown that thrombin generated in plasma during coagulation is inhibited by HCII when dermatan sulfate is present, thus explaining the anticoagulant effect of dermatan sulfate that has been observed in vitro (10, 12).

EXPERIMENTAL PROCEDURES

Materials—Benzoyl-He-Glu-Gly-Arg-p-nitroanilide (S-2222), pyro-Glu-Gly-Arg-p-nitroanilide (S-2444), Val-Leu-Lys-p-nitroanilide (S-2561), Pro-Phe-Arg-p-nitroanilide (S-2302), and Phe-Pip-Arg-p-nitroanilide (S-2228) were purchased from Helena Laboratories; succinyl-Ala-Ala-Pro-Phe-p-nitroanilide from VEGA Biochemicals; succinyl-Ala-Ala-Ala-p-nitroanilide from Sigma; and tosyl-Gly-Pro-Arg-p-nitroanilide (Cromozyme TH) from Boehringer Mannheim. Heparin from porcine intestinal mucosa was obtained from Abbott Laboratories. Porcine skin dermatan sulfate was obtained from Sigma and was treated with nitrous acid prior to use to remove contaminating heparin (5, 10). Sodium[125I]iodide (16.8 Ci/mg) was purchased from Amersham.iodoge, was purchased from Pierce. Prothrombin-deficient plasma containing ~2% of the normal concentration of prothrombin was purchased from George King Biologica. Normal plasma was obtained from blood (4.5 ml) drawn into evacuated tubes containing 0.5 ml of 0.129 M buffered sodium citrate (Vacutainer No. 6418, Becton-Dickinson). Activated partial thromboplastin reagent was obtained from Hyland Laboratories. Rabbit brain thromboplastin was obtained from Ortho Diagnostics and was reconstituted with water according to the manufacturer. Human brain thromboplastin (13) was obtained from Dr. George Broze, Washington University. Rabbit brain cephalin was purchased from Sigma. Polybrene (1,3-dimethyl-1,3-diazanoundecamethylene polymethobromide) was obtained from Aldrich.

Proteins—Human HCII and thrombin were purified as previously described (4). Human factor XIIa (14) was prepared by Dr. Allen Kaplan, State University of New York, Stony Brook. Human factor Xa (15) was obtained from Dr. Paul Bajaj, University of California, San Diego. Human coagulation factors VIIa (13), IX, X, Xa (16), and activated protein C (17) were obtained from Drs. Hatem Salem, George Broze, and Joseph Miletich, Washington University. Factor IX (69 μM) was converted to IXa by incubation with 40 nM factor IXa for 2 h at 37 °C in buffer containing 5 mM CaCl2, 0.05 M NaCl, 0.02 M Tris-HCl, pH 7. Tissue plasminogen activator derived from cultured human melanoma cells (18) was obtained from Dr. Desiré Collen, University of Leuven. Human urokinase was the product of Winthrop Laboratories. Glu-plasminogen II was purified from human plasma by the method of Deutsch and Mertz (19) and the zymegen

*This research was supported by grants from the National Institutes of Health (HL-27589) and the Monsanto Co. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡Recipient of National Institutes of Health Career Development Award HL-01079.

The abbreviations used are: HCII, heparin cofactor II; ATIII, antithrombin III; TPA, tissue plasminogen activator; γ-NGF, the peptide subunit of nerve growth factor; EGF-BF, epidermal growth factor-binding protein; and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

D. M. Tollefsen and C. A. Pestka, manuscript submitted.
Continuous recording of the absorbance at 405 nm. Alternatively, employed in the experiment. The following substrates were used: 0.1 mM plasmin; 0.6 mM S-2444 for urokinase; 0.2 mM succinyl-Ala-Ala-Pro-glycan was removed by centrifugation if necessary, and the absorbance was determined from a plot of optical density against time.

The rate of hydrolysis of the substrate was determined from a standard curve of absorbance versus time. Hydrolysis of the substrate was terminated after 3 min by addition of 10% trichloroacetic acid (5% final concentration), the mixture was centrifuged for 10 min at 4 °C, and the supernatant was assayed for protease activity.

Ectodipeptidase—SDS-PAGE was performed with 7.5% gels and the Laemmli buffer system under nonreducing conditions. Autoradiography was performed as described previously. Molecular weight standards were obtained from BioRad.

TABLE I

<table>
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<th>Propeptide</th>
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<th>[HCII]</th>
<th>Incubation time</th>
<th>[Dermatan sulfate]</th>
<th>Activity remaining after incubation with</th>
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<td>nM</td>
<td></td>
<td></td>
<td>ug/ml</td>
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<td>nM</td>
<td>min</td>
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* Averages of duplicate determinations.

** ND, not determined.

** Averages of duplicate determinations.
Inhibition of Thrombin by HCII in Recalcified Plasma—We have previously shown that thrombin combines with HCII to form a 96,000-dalton complex that is stable during SDS-PAGE (3, 4). To determine whether HCII inhibits thrombin or other proteases as they are generated during coagulation, tracer $^{125}$I-HCII was added to citrate-anticoagulated plasma, and coagulation was initiated by addition of CaCl$_2$. After 1 h, the plasma was analyzed by SDS-PAGE and autoradiography (Fig. 1, lanes E–G). Coagulation occurred in the absence of heparin or dermatan sulfate, and none of the $^{125}$I-HCII was detected in higher molecular weight complexes (lane E). However, coagulation did not occur in the presence of 67 µg/ml of dermatan sulfate, and densitometry revealed that ~13% of the label was present in a 96,000-dalton band (lane G) which co-migrated with the complex formed by incubation of an excess of purified thrombin with $^{125}$I-HCII (lane A). Less than 1% of the label was present in the complex when prothrombin-deficient plasma was substituted for normal plasma in the incubation (not shown). Coagulation also did not occur in the presence of 5 units/ml of heparin. In this case, none of the $^{125}$I-HCII was detected in the 96,000-dalton complex (lane F).

This result is consistent with previous experiments which demonstrated that $^{125}$I-thrombin is preferentially inhibited by ATIII in undiluted plasma at similar concentrations of heparin (3). Control incubations from which the CaCl$_2$ was omitted did not contain labeled complexes regardless of whether heparin or dermatan sulfate was present (lanes B–D). These experiments indicate that thrombin generated in plasma by activation of the intrinsic coagulation pathway is inhibited by HCII in the presence of dermatan sulfate.

Inhibition of Thrombin by HCII in Plasma Activated by Tissue Factor or Kaolin—In an attempt to detect complexes of HCII with proteases other than thrombin, prothrombin-deficient plasma containing $^{125}$I-HCII was incubated for 1 h at 37 °C with CaCl$_2$, phospholipids, and a source of tissue factor (human brain thromboplastin) to activate factor VII or kaolin (activated partial thromboplastin reagent) to activate factor XII and kallikrein. In neither case was any of the $^{125}$I-HCII detected in complexes (Fig. 1, lanes H and K). Identical results were obtained in the presence of 5 units/ml of heparin (lanes I and L). When incubations were repeated in the presence of 67 µg/ml of dermatan sulfate, a trace amount of the label was present in a band corresponding to the thrombin-HCII complex (lanes J and M). No other complexes were observed. When the exposure times of the autoradiographs in Fig. 1 were extended from 1 to 20 h to increase the sensitivity of the experiments, we observed numerous additional bands representing as a whole 0.1% of the total radioactivity present. Because there were no significant differences between the additional bands and the pattern of a gel containing $^{125}$I-HCII alone (not shown), the bands were considered to represent trace contaminants in the HCII preparation.

**Inhibition of Purified Proteases by HCII**—We assayed fourteen purified proteases for activity after incubation with a molar excess of HCII (Table I). The concentrations of protease and HCII were determined primarily according to the sensitivity of the assay for the protease. Incubation times were long in comparison to the $t_{1/2}$ for inhibition of thrombin by HCII (e.g. $t_{1/2} = 8$ s in the presence of 50 nM HCII and 0.5 unit/ml of heparin; Ref. 4). Under the conditions of each experiment, 20% inhibition of the protease would indicate a second-order rate constant $\leq 5 \times 10^8$ M$^{-1}$ min$^{-1}$ (i.e. 2000 times less than the rate constant for inhibition of thrombin by HCII in the presence of dermatan sulfate; see “Discussion”). Heparin or dermatan sulfate were present at concentrations previously determined to accelerate the inhibition of thrombin by HCII (4, 5). In addition, controls were performed to determine the effects of heparin and dermatan sulfate alone on protease activity. As shown in Table I, 22% of thrombin activity remained after a 20-min incubation with 150 µM HCII, while <2% activity remained in incubations that also included heparin or dermatan sulfate. The second-order rate constant calculated for inhibition of thrombin by HCII alone in this experiment was $5 \times 10^8$ M$^{-1}$ min$^{-1}$, as previously reported (4). Rate constants for inhibition of thrombin by HCII in the presence of heparin or dermatan sulfate could not be determined accurately from the data in Table I, but in both cases were $>1.3 \times 10^9$ M$^{-1}$ min$^{-1}$ (see “Discussion”). In contrast, HCII did not inhibit significantly coagulation factors VIIa, IXa, Xa, XIa, XIIa, kallikrein, activated protein C, plasmin, urokinase, TPA,$^3$ or γ-NGF. Activated protein C and TPA were moderately inhibited by dermatan sulfate and heparin, respectively, but there was no further inhibition in either case when HCII was also present. Leukocyte elastase was partially inhibited during a 60-min incubation with 1.78 µM HCII alone or with dermatan sulfate; in both cases the rate constants were $5 \times 10^8$ M$^{-1}$ min$^{-1}$. Although elastase was moderately inhibited by heparin alone, heparin appeared to

$^3$ HCII was incubated with TPA alone (data not shown) or in the presence of fibrin as described under “Experimental Procedures” (data in Table I).

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**RESULTS**

**Fig. 1. Incorporation of $^{125}$I-HCII into complexes in plasma during coagulation.** Reagents were brought to a final volume of 150 µl with 0.15 M NaCl, 0.02 M Tris-HCl, 1 mg/ml bovine serum albumin, pH 7.4, and incubated for 1 h at 37 °C in glass (lanes B–G) or polypropylene (lanes A and H–M) tubes. Each incubation contained 7.5 nM $^{125}$I-HCII along with the following additional reagents: lane A, 15 µM thrombin; lane B, 25 µl of normal plasma; lane C, same as B plus 5 units/ml heparin; lane D, same as B plus 67 µg/ml dermatan sulfate; lane E, 25 µl of normal plasma and 6 mM CaCl$_2$; lane F, same as E plus 5 units/ml heparin; lane G, same as E plus 67 µg/ml dermatan sulfate; lane H, 25 µl of prothrombin-deficient plasma, 13 mM CaCl$_2$, and 100 µl of human brain thromboplastin; lane I, same as H plus 5 units/ml heparin; lane J, same as H plus 67 µg/ml dermatan sulfate; lane K, 25 µl prothrombin-deficient plasma, 8 mM CaCl$_2$, and 50 µl of activated partial thromboplastin reagent; lane L, same as K plus 5 units/ml heparin; lane M, same as K plus 67 µg/ml dermatan sulfate. At the end of the incubation period, 5–10 µl of each reaction mixture were subjected to SDS-PAGE. An autoradiograph of the gel exposed for 1 h at -70 °C is shown. The positions of molecular weight standards and of the 96,000-dalton thrombin-HCII complex (→) are indicated.
protect the protease from further inhibition by HCII. EGF-BP was inhibited partially by dermatan sulfate and to a slightly greater extent when both dermatan sulfate and HCII were present; in the latter case the calculated rate constant was \(1 \times 10^5 \text{ M}^{-1} \text{min}^{-1}\).

Inhibition of Cathepsin G by HCII—Because HCII appeared to inhibit cathepsin G at a significant rate in the presence of dermatan sulfate (Table I), the time course of inhibition was studied in more detail (Fig. 2). In the absence of heparin or dermatan sulfate, inhibition by 1.25 \(\mu\)M HCII occurred with a \(t_{1/2} = 24\) min (\(k = 1.4 \times 10^4 \text{ M}^{-1} \text{min}^{-1}\)). Although dermatan sulfate alone decreased cathepsin G activity 20–30% (Table I), it increased the rate of inhibition by HCII 6-fold (\(t_{1/2} = 4\) min; \(k = 8.4 \times 10^4 \text{ M}^{-1} \text{min}^{-1}\)). In contrast, heparin decreased the activity of cathepsin G 50–60%, and it appeared to prevent further inhibition of the protease by HCII.

**DISCUSSION**

The purpose of this investigation was to identify enzymes that HCII can inhibit, and thereby to arrive at a hypothesis concerning the physiological function of HCII. We have tested various serine proteases, including all of those currently known to be involved in coagulation and fibrinolysis (28), leukocyte cathepsin G (21) and elastase (20), and the peptide subunits of nerve growth factor (23) (\(\gamma\)-NGF) and epidermal growth factor (24) (EGF-BP). Of the enzymes tested other than thrombin, only leukocyte cathepsin G was inhibited at a significant rate. However, the calculated second-order rate constant for inhibition of cathepsin G by HCII in the presence of dermatan sulfate was \(~40,000\)-fold less than the rate constant reported for inhibition of cathepsin G by \(\alpha_1\)-antichymotrypsin (29). Therefore, inhibition of cathepsin G by HCII is unlikely to occur in vivo. In contrast, HCII inhibits thrombin with rate constants of \(6.4 \times 10^8 \text{ M}^{-1} \text{min}^{-1}\) in the presence of 250 \(\mu\)g/ml of dermatan sulfate and \(4.0 \times 10^8 \text{ M}^{-1} \text{min}^{-1}\) in the presence of 10 units/ml of heparin (5). Rate constants of this magnitude are characteristic of inhibition reactions that are likely to be "physiological" (30).

Our data indicate that the protease specificity of HCII is more restricted than that of other plasma protease inhibitors, including ATIII (9), \(\alpha_1\)-proteinase inhibitor (30), \(\alpha_2\)-antiplasmin (31), and \(\alpha_2\)-macroglobulin (32), each of which can inhibit several of the proteases that we have tested. In addition, the lack of inhibition of \(\gamma\)-NGF, EGF-BP, plasmin, and urokinase distinguishes HCII from the cellular protease inhibitors termed "protease nexins" (33). We have also found that, in the presence of dermatan sulfate, HCII binds thrombin as it is being generated in plasma during coagulation. Thus, inhibition of thrombin by HCII appears to explain the anticoagulant activity of dermatan sulfate that has been observed in vitro (10, 12) and may also explain the antithrombotic effect observed in vivo after the administration of exogenous dermatan sulfate (34). In addition, HCII may inhibit other effects of thrombin, including platelet aggregation and secretion (35), chemotaxis (36), and mitogenesis (37), under appropriate circumstances.

Rapid inhibition of thrombin by HCII in vivo probably occurs only in the immediate vicinity of proteoglycans which contain oligosaccharide sequences that bind HCII (4, 8). Similarly, ATIII requires specific oligosaccharide sequences for maximum activity (9). HCII and ATIII may become activated in different environments, since there is evidence that different heparin molecules activate HCII and ATIII (38) and that dermatan sulfate only activates HCII (5). Dermatan sulfate comprises 60–70% of the glycosaminoglycans in the intima and media of large arteries (39), in addition to being present in skin, heart valves, and tendons (40). A small amount of dermatan sulfate is also synthesized by cultured endothelial cells (41). Whether the glycosaminoglycans present in these locations contain the proper sequences to activate HCII remains to be determined.

**Acknowledgments**—We thank Dr. Allen Kaplan for performing the factor XIIa assays and Drs. Paul Bajaj, George Broze, Dèsiré Collen, Thomas Maciag, Joseph Miletich, Hatem Salem, and Robert Senior for providing proteases and other reagents.

**REFERENCES**

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8, 859–867