Substitution of Arginine for Leu\textsuperscript{444} in the Reactive Site of Heparin Cofactor II Enhances the Rate of Thrombin Inhibition*

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Heparin cofactor II (HCII), a member of the “serpin” family of serine protease inhibitors, is a 65,600-Da plasma glycoprotein that inhibits thrombin and chymotrypsin. The rate of thrombin inhibition is stimulated ~1000-fold by heparin or dermatan sulfate. Thrombin and chymotrypsin cleave the Leu\textsuperscript{444}-Ser\textsuperscript{445} bond (designated P\textsubscript{1}-P\textsubscript{2}) in the reactive site of HCII, forming a stable equimolar complex in which the protease is inactive. In this study, we have determined the effects of substituting an arginine for Leu\textsuperscript{444} in recombinant HCII (rHCII). The rHCII was expressed in Escherichia coli and partially purified by heparin-Sepharose chromatography. Apparent second-order rate constants (k\textsubscript{2}) for inhibition of thrombin, coagulation factor Xa, kallikrein, plasmin, and chymotrypsin by rHCII were determined using appropriate chromogenic substrates. In the absence of a glycosaminoglycan, rHCII(Leu\textsuperscript{444} → Arg) inhibited thrombin at a 98-fold higher rate (k\textsubscript{2} = 6.2 \times 10\textsuperscript{6} M\textsuperscript{-1} min\textsuperscript{-1}) than native rHCII (k\textsubscript{2} = 6.3 \times 10\textsuperscript{4} M\textsuperscript{-1} min\textsuperscript{-1}). Dermatan sulfate accelerated thrombin inhibition by both forms of rHCII, but the maximum rate constant in the presence of dermatan sulfate was only 2-fold higher for rHCII(Leu\textsuperscript{444} → Arg) (k\textsubscript{2} = 5.3 \times 10\textsuperscript{6} M\textsuperscript{-1} min\textsuperscript{-1}) than for native rHCII (k\textsubscript{2} = 2.2 \times 10\textsuperscript{6} M\textsuperscript{-1} min\textsuperscript{-1}). Heparin was less effective than dermatan sulfate in stimulating both forms of rHCII. Factor Xa, kallikrein, and plasmin were inhibited more rapidly and chymotrypsin more slowly by rHCII(Leu\textsuperscript{444} → Arg) than by native rHCII. These effects are qualitatively similar to those observed with the natural mutant α1-antitrypsin Pittsburgh (Met\textsuperscript{358} → Arg at the P\textsubscript{1} position) and strengthen the hypothesis that the P\textsubscript{1} residue is a major determinant of protease specificity in the serpins. Furthermore, the rapid rate of inhibition of thrombin by rHCII(Leu\textsuperscript{444} → Arg) in the absence of heparin or dermatan sulfate suggests that this variant may be useful as a therapeutic agent.

The anticoagulant activities of glycosaminoglycans are mediated by antithrombin (AT) and heparin cofactor II (HCII), which are members of the “serpin” family of serine protease inhibitors (1). HCII inhibits thrombin but has no activity against other proteases involved in coagulation or fibrinolysis (2). In contrast, AT inhibits all of the proteases of the intrinsic coagulation pathway as well as the fibrinolytic protease plasmin (3, 4). Heparin and dermatan sulfate increase the rate of thrombin inhibition by HCII ~1000-fold by providing a catalytic template to which both the inhibitor and the protease bind (5, 6). Although heparin catalyzes protease inhibition by both HCII and AT, the anticoagulant effect of dermatan sulfate is mediated exclusively by HCII (5).

HCII forms a stable 1:1 complex with thrombin or chymotrypsin in which the protease is inactive. During complex formation, both thrombin and chymotrypsin attack the reactive site Leu\textsuperscript{444}-Ser\textsuperscript{445} peptide bond (designated P\textsubscript{1}-P\textsubscript{2}) near the COOH-terminal end of HCII (7–9). The resulting complex does not dissociate when heated at 100 °C with sodium dodecyl sulfate, suggesting that under these conditions the two proteins become linked by a covalent bond. The bond is presumed to be an ester linkage between the scissile hydroxyl group in the active site of the protease and the carbonyl group of Leu\textsuperscript{444} in HCII. Therefore, HCII can be thought of as a pseudosubstrate for these proteases.

The P\textsubscript{1} residue in the reactive site of a serpin appears to play a major role in determining the relative rates of inhibition of various proteases (10). Evidence for this was derived from analysis of the variant α1-antitrypsin Pittsburgh, which was discovered in a child who had a fatal bleeding disorder. The variant contained an arginine residue in place of Met\textsuperscript{358} at the P\textsubscript{1} position of the inhibitor (11). This substitution resulted in markedly increased rates of inhibition of several proteases, including thrombin, factor Xa, factor Xla, kallikrein, and plasmin, which cleave Arg-X peptide bonds in their natural substrates (12–14). A reciprocal decrease in the rate of inhibition of neutrophil elastase, the principal target protease of native α1-antitrypsin, was also noted. In the present study, we have tested the hypothesis that substitution of arginine for Leu\textsuperscript{444} at the P\textsubscript{1} position of HCII would affect the rates of inhibition of thrombin and other proteases in a manner similar to that observed with α1-antitrypsin Pittsburgh.

EXPERIMENTAL PROCEDURES

Materials—Chromogenic substrates were purchased from the following sources: tosyl-Gly-Pro-Arg-p-nitroanilide (Chromozym TH), Boehringer Mannheim; N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, Sigma; methoxy carbonyl cyclohexylglycyl-Gly-Arg-p-nitroanilide.
(Spectrozyme FXa), American Diagnostica; Pro-Phe-Arg-p-nitroanilide (S-2302) and pyroglutamyl-Pro-Lys-p-nitroanilide (S-2366), KabiVitrum. Thrombin and HCFI derived from human plasma were prepared and assayed as previously described (15). Human coagulation factor Xa was provided by Dr. George Wroble, Washington University. Human plasmin was provided by Dr. Francis J. Castellino, University of Notre Dame. Human chymotrypsin and plasma kallikrein were purchased from Sigma. Affinity purified rabbit anti-HCFI antibodies were prepared and labeled with Na[125]I as described previously (16). Bovine lung heparin and porcine skin dermanan sulfate were purchased from Du Pont-New England Nuclear. The Protein Chemistry Facility of Washington University prepared calf thymus deoxyguanucleic acid (dG) containing deoxyribonucleic acid (dA).

The reagents used for oligonucleotide-directed mutagenesis were purchased from Amersham Corp. Restriction enzymes and DNA modification reagents were obtained from Amersham, New England Biolabs, and Bethesda Research Laboratories. Reagents for the T4 polymerase chain reaction were purchased from PerkinElmer Cetus. DNA sequencing was performed using T7 DNA polymerase from U. S. Biochemical Corp. Deoxyadenosine [α-35S]dATP was obtained from Du Pont-New England Nuclear. The Protein Chemistry Facility of Washington University provided calf thymus deoxyguanucleic acid (dG) containing deoxyribonucleic acid (dA).

Construction of the pMON-HCFI Expression Vector—Previously, the PstI-HindII fragment of the HCFI cDNA was inserted into pMON-5840 to yield a construct in which the NH2-terminal 18 amino acid residues of mature plasma HCFI were replaced by the sequence Met-Ala-(15). Although the resulting inhibitor was active by several criteria, we wanted to obtain a full-length HCFI for the present studies. In addition, we constructed unique Sall and BstEII restriction sites for cassette mutagenesis of the reactive site. The Sall site in the parent vector pMON-5840 was deleted to form pMON-5840(SalI) by digestion with Sall, treatment with mung bean exonuclease, and ligation. A full-length cDNA for HCFI was isolated previously from a human fetal liver library in Agt11 (16). Oligonucleotide-directed mutagenesis of the cDNA was performed in M13mp18 containing the noncoding strand by the method of Nakamaye and Eckstein (19). The codon for Val17 was altered (GTC + GTT) to remove a SalI site and create a BstEII site. Cassette mutagenesis between BstEII and SalI was performed to create the reactive-site mutant pMON-HCFI(Leu4 + Arg). The open rectangle denotes the recA promoter (PrecA) and the gene 10 leader (G10L) ribosome-binding site (16).

RESULTS

Expression Vectors for HCFI—A diagram of the HCFI expression vector (pMON-HCFI) is shown in Fig. 1. The mRNA produced by this vector contains an initiator Met codon followed by the nucleotide sequence encoding the NH2-terminal 18 amino acids at the translation initiation and termination sites and in the 3′ untranslated sequence derived from the HCFI cDNA. The recA promoter (PrecA) and the gene 10 leader (G10L) ribosome-binding site (16).

The concentration of HCFI was determined by a slot-blot immunonassay. Various amounts of the partially purified HCFI were blotted onto nitrocellulose, and HCFI was detected with [125]I-labeled anti-HCFI antibodies (16). After autoradiography, the bands were scanned with a densitometer and the area under each peak was determined (LKB Ultrascan). A standard curve constructed with known amounts of plasma HCFI was linear from 2.5 to 90 ng. The standard deviation of multiple determinations of plasma HCFI using this method was ≤20%.

Inhibition of Proteases by Human HCFI—Protease inhibition by HCFI was determined by incubating 50 μl of the partially purified HCFI (or the corresponding heparin-Sepharose fractions of a control lysate from cells transformed with pMON-5840) with 5 μl of the protease and 5 μl of either glycine-sodiumglycinate or water in a disposable polypropylene cuvette at room temperature. Final concentrations of the proteases were as follows: 15 nM thrombin, 21 nM chymotrypsin, 16 nM factor Xa, 24 nM plasmin, and 10 nM kallikrein. After a specified period of time from 5 s to 150 min, 300-500 μl of the appropriate substrate in the 100 μl reaction mixture was added, and the absorbance at 405 nm was recorded continuously for 100 s. The rate of change of absorbance was proportional to the concentration of active protease remaining in the incubation. The amounts added and concentrations of each substrate were as follows: 500 μl of 0.1 mM Chromozym TH (thrombin), 300 μl of 0.5 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (chymotrypsin), 400 μl of 0.125 mM S-2366 (factor Xa), 300 μl of 0.67 mM S-2302 (plasmin), and 300 μl of 0.4 mM S-2302 (kallikrein).

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Protease Inhibition by rHCII in the Absence of a Glycosaminoglycan—The time courses of inhibition of thrombin, factor Xa, kallikrein, plasmin, and chymotrypsin by native rHCII and rHCII(Leu$^{44} \rightarrow$ Arg) in the absence of a glycosaminoglycan are indicated in Fig. 3. The incubations were performed under pseudo first-order conditions, such that the initial concentration of rHCII was greater than the concentration of protease. The pseudo first-order rate constants ($k_t$) for protease inhibition were determined by fitting the data to the following equation: $k_t \cdot t = \ln([P]_0/[P]_t)$, in which $[P]_0$ = initial protease activity and $[P]_t$ = protease activity at time $t$. The second-order rate constants ($k_{st}$) were then calculated by dividing $k_t$ by the initial rHCII concentration. The rate constants are summarized in Table I. We observed that rHCII(Leu$^{44} \rightarrow$ Arg) inhibits thrombin almost 100 times more rapidly than native rHCII in the absence of a glycosaminoglycan. Furthermore, rHCII(Leu$^{44} \rightarrow$ Arg) inhibits factor Xa and kallikrein, while inhibition of these proteases by native rHCII is undetectable. Plasmin is inhibited very slowly by rHCII(Leu$^{44} \rightarrow$ Arg) but not by native rHCII. In contrast to all of the above mentioned proteases, chymotrypsin is inhibited by native rHCII but not by rHCII(Leu$^{44} \rightarrow$ Arg).

Inhibition of Thrombin by rHCII in the Presence of Dermatan Sulfate or Heparin—The time course of inhibition of thrombin was determined in the presence of various concentrations of dermatan sulfate or heparin. The second-order rate constants derived from these experiments are shown in Fig. 4. Maximum rate constants were observed at 25-100 µg/ml of either glycosaminoglycan (Table I). Dermatan sulfate produces approximately a 3500-fold increase in the rate constant for thrombin inhibition by native rHCII but has a much smaller effect on the rate constant obtained with rHCII(Leu$^{44} \rightarrow$ Arg). Thus, rHCII(Leu$^{44} \rightarrow$ Arg) inhibits thrombin only about twice as fast as native rHCII in the presence of dermatan sulfate. Heparin increases the rate constant for thrombin inhibition by native rHCII about 40-fold but has a minimal effect on the rate constant obtained with rHCII(Leu$^{44} \rightarrow$ Arg).

Inhibition of Chymotrypsin, Factor Xa, Plasmin, and Kallikrein by rHCII in the Presence of Dermatan Sulfate or Heparin—Dermatan sulfate does not significantly increase the rate of inhibition of chymotrypsin by native rHCII, nor does it increase the rate of inhibition of factor Xa, plasmin, or kallikrein by rHCII(Leu$^{44} \rightarrow$ Arg) (Table I). Heparin (100 µg/ml) appears to decrease the rates of inhibition of factor Xa, plasmin, and kallikrein by rHCII(Leu$^{44} \rightarrow$ Arg).

**DISCUSSION**

The target protease specificity of a serpin is strongly influenced by the amino acid residue at the $P_1$ position of the reactive site, as expected if the reactive site resembles a substrate for the protease (10). For example, the presence of Met$^{58}$ at the $P_1$ position of $\alpha_1$-antitrypsin is consistent with the substrate specificity of its major target protease, neutrophil elastase, which preferentially cleaves the peptide bond following a medium sized hydrophobic amino acid (23). Amidohydrolysis of the P, methionine residue of $\alpha_1$-antitrypsin both decreases the rate of inhibition of elastase and increases the rate of inhibition of trypsin and plasmin (24). Similarly, the natural mutant (Met$^{58} \rightarrow$ Arg) $\alpha_1$-antitrypsin Pittsburgh
is a poor inhibitor of elastase but a potent inhibitor of thrombin and certain other proteases which preferentially attack Arg-X peptide bonds (12-14).

The reactive site of HCII was identified by Griffith and co-workers (7, 8) who isolated the peptide released from the inhibitor when it reacts with thrombin or chymotrypsin. Thrombin and chymotrypsin cleave HCII at precisely the same peptide bond near the COOH-terminus of the protein, yielding a peptide 36 amino acids in length with an NH2-terminal serine residue. The peptide remains noncovalently attached to the HCII-protease complex but can be isolated under denaturing conditions by reverse-phase chromatography. The peptide released from HCII when it is inactivated by a protease from Echis carinatus is 1 residue longer (i.e., 37 amino acids) than the peptide released by thrombin, having a leucine residue at its NH2 terminus (9). These observations, in conjunction with the cDNA sequence of HCII (16, 25), identified the Leu44-Ser445 (P1-P1') bond as the reactive site of HCII.

The presence of leucine at the P1 position appears to explain in part the protease specificity of HCII. HCII inhibits chymotrypsin more rapidly than it inhibits thrombin in the absence of a glycosaminoglycan, with reported second-order rate constants of $1.8 \times 10^6$ M$^{-1}$ min$^{-1}$ for chymotrypsin (7) and $5.0 \times 10^5$ to $5.0 \times 10^7$ M$^{-1}$ min$^{-1}$ for thrombin (7, 18). In addition, HCII fails to inhibit a variety of other proteases that preferentially cleave substrates following a basic amino acid residue (2). Our observations with native rHCII (Fig. 3 and Table I) are consistent with these findings. Furthermore, substitution of arginine for Leu44 at the P1 position of rHCII increases the rate of inhibition of thrombin 100-fold while reducing the rate of inhibition of chymotrypsin to an undetectable level. rHCII(Leu44 $\rightarrow$ Arg) also has the ability to inhibit factor Xa, plasmin, and kallikrein, proteases that are not inhibited by native rHCII or plasma HCII. These results indicate the importance of the P1 residue in determining the rate of protease inhibition by HCII in the absence of a glycosaminoglycan.

The difference between the rates of inhibition of chymotrypsin and thrombin (3- to 4-fold in the absence of a glycosaminoglycan) is perhaps smaller than one would expect if the P1 residue were the sole determinant of the rate. Several observations suggest that Pro445 at the P1 position may facilitate the thrombin-HCII reaction. For example, proline is

### Table I

<table>
<thead>
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<th>Protase</th>
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<td>-Glycosaminoglycan +Deratan sulfate +Heparin</td>
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<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>$\leq 1 \times 10^6$</td>
<td>ND</td>
</tr>
<tr>
<td>Plasmin</td>
<td>$\leq 1 \times 10^6$</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Average of three determinations.
* Average of two determinations.
* Single determination.
* ND, not determined.

Fig. 3. Inhibition of proteases by native rHCII and rHCII(Leu$^{44}$ $\rightarrow$ Arg). Heparin-Sepharose column eluates (90 μl) of native rHCII (142 nM), rHCII(Leu$^{44}$ $\rightarrow$ Arg) (76 nM), or pMON-5840 control were incubated with thrombin (15 nM), chymotrypsin (21 nM), factor Xa (16 nM), plasmin (24 nM), or kallikrein (10 nM) in a total volume of 100 μl at the final concentrations indicated above. After various times of incubation, the remaining protease activity was determined by adding the appropriate chromogenic substrate and measuring ΔA405/min as described under "Experimental Procedures." The percent protease activity was calculated from the ratio of final to initial protease activity.
The importance of the P₁ residue is less apparent when the P₂ residue is Leu (14). However, when the P₃ residue is also Leu, the rate constant for inhibition of thrombin by rHCI₁(Leu₄⁴⁴ → Arg) is -3-fold less than that reported for α₁-antitrypsin Pittsburgh (14, 28) (Table I). The presence of 10-40 μM α₁-antitrypsin Pittsburgh in the plasma of the patient from whom the variant was isolated caused severe bleeding associated with prolonged in vitro clotting times (activated partial thromboplastin time >150 s; thrombin time >60 s) (11, 33). It is likely that high concentrations of rHCI(Leu₄⁴⁴ → Arg) would produce a similar effect, while lower concentrations might be sufficient to prevent the development or extension of a thrombus. Because rHCI(Leu₄⁴⁴ → Arg) inhibits plasmin ~1000-fold less rapidly than α₁-antitrypsin Pittsburgh (14), rHCI(Leu₄⁴⁴ → Arg) would theoretically produce less of an antifibrinolytic effect and thus be preferable to α₁-antitrypsin Pittsburgh as an antithrombotic agent. α₁-Antitrypsin Pittsburgh has been considered for the treatment of hereditary angioedema (C1-inhibitor deficiency) and septic shock because of its ability to inhibit thrombin rapidly in the absence of heparin. The rate constant for inhibition of thrombin by rHCI(Leu₄⁴⁴ → Arg) is ~3-fold less than that reported for α₁-antitrypsin Pittsburgh (14). We also found that heparin causes a 40-fold increase in thrombin inhibition with native rHCI₁, but only a 3-fold increase with rHCI(Leu₄⁴⁴ → Arg). We have consistently observed that the maximum rate constant for thrombin inhibition by native rHCI₁ in the presence of heparin (2.4 × 10⁻⁶ M⁻¹ min⁻¹) is about 2 orders of magnitude less than that observed with dermatan sulfate (2.3 × 10⁻⁵ M⁻¹ min⁻¹). In comparison, the maximum rate constants obtained with purified plasma HClI are similar in the presence of the two glycosaminoglycans, i.e. 6.4 × 10⁻⁵ M⁻¹ min⁻¹ with dermatan sulfate and 3.8 × 10⁻⁵ M⁻¹ min⁻¹ with heparin (5). Although we cannot explain this discrepancy at the present time, preliminary experiments suggest that rHCI may be more susceptible to proteolytic inactivation than plasma HClI in the presence of heparin. * This effect may be similar to that reported by Olson (32), who showed that under certain conditions heparin converts AT from an inhibitor to a substrate for thrombin.

The peptide sequence Gly-Arg-Ser in thrombin substrate 1 (15) is known to be a binding site for heparin (31). However, the minimum heparin concentration required to stimulate thrombin inhibition by rHCI(Leu₄⁴⁴ → Arg) is 10⁻⁶ M (Fig. 4) whereas a 10⁻⁴ M concentration is needed for rHCI (7). On the other hand, one might have predicted that glycosaminoglycans would stimulate inhibition of factor Xa, plasmin, and kallikrein by rHCI(Leu₄⁴⁴ → Arg), since inhibition of these proteases by AT is stimulated by heparin (3, 4). Unexpectedly, heparin appears to decrease the rate of inhibition of kallikrein by rHCI(Leu₄⁴⁴ → Arg). Although the mechanism of this effect is unknown, one possibility is that rHCI(Leu₄⁴⁴ → Arg) is a substrate for kallikrein in the presence of heparin and is thereby inactivated.

Heparin is commonly employed in the prophylaxis and treatment of venous thrombosis and pulmonary embolism, but its use is sometimes complicated by severe bleeding or thrombocytopenia. Therefore, alternatives to heparin for short-term anticoagulation would be desirable. rHCI(Leu₄⁴⁴ → Arg) might be considered in this regard because of its ability to inhibit thrombin rapidly in the absence of heparin. The rate constant for inhibition of thrombin by rHCI(Leu₄⁴⁴ → Arg) is ~3-fold less than that reported for α₁-antitrypsin Pittsburgh (14, 28) (Table I). The presence of 10-40 μM α₁-antitrypsin Pittsburgh in the plasma of the patient from whom the variant was isolated caused severe bleeding associated with prolonged in vitro clotting times (activated partial thromboplastin time >150 s; thrombin time >60 s) (11, 33). It is likely that high concentrations of rHCI(Leu₄⁴⁴ → Arg) would produce a similar effect, while lower concentrations might be sufficient to prevent the development or extension of a thrombus. Because rHCI(Leu₄⁴⁴ → Arg) inhibits plasmin ~1000-fold less rapidly than α₁-antitrypsin Pittsburgh (14), rHCI(Leu₄⁴⁴ → Arg) would theoretically produce less of an antifibrinolytic effect and thus be preferable to α₁-antitrypsin Pittsburgh as an antithrombotic agent. α₁-Antitrypsin Pittsburgh has been considered for the treatment of hereditary angioedema (C1-inhibitor deficiency) and septic shock because of its ability to inhibit kallikrein and thereby prevent the generation of bradykinin (12, 13), and it has been shown to prolong survival in experimental Gram-negative septicemia (31, 34). rHCI(Leu₄⁴⁴ → Arg) inhibits kallikrein ~30-fold more slowly than α₁-antitrypsin Pittsburgh and, therefore, one would not expect it to be as effective in the treatment of septic shock. The in vivo properties and therapeutic use of rHCI(Leu₄⁴⁴ → Arg) warrant investigation in animal models.

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