Heparin cofactor II (HCII) inhibits thrombin by forming a stable 1:1 complex. Heparin and dermatan sulfate increase the rate of complex formation 1000-fold. Mutation of leucine 444 to arginine at the P1 position of recombinant HCII (rHCII) increases the rate of inhibition of thrombin ~100-fold in the absence of a glycosaminoglycan (Derechin, V. M., Blinder, M. A., and Tollefsen, D. M. (1990) J. Biol. Chem. 265, 5623–5628). We now report that heparin facilitates dissociation of the thrombin–rHCII(L444R) complex. In the presence of heparin, thrombin is inhibited rapidly and completely by a 35-fold molar excess of rHCII(L444R), but subsequently ~50% of the thrombin activity reappears with a $t_{1/2}$ of ~20 min. At higher ratios of rHCII(L444R) to thrombin, the reappearance of thrombin activity is delayed and the final plateau of activity is decreased. Electrophoretic analysis indicates that proteolysis of excess rHCII(L444R) precedes the reappearance of thrombin activity. Addition of heparin at longer intervals after formation of the thrombin–rHCII(L444R) complex causes a progressive decrease in the thrombin plateau, suggesting that in the absence of heparin the complex is slowly converted to a non-dissociable form. By contrast to heparin, dermatan sulfate does not facilitate dissociation of the thrombin–rHCII(L444R) complex. Our findings indicate that the P1 residue of HCII affects not only the rate of inhibition of thrombin but also the stability of the resulting complex.

When HCII is bound to certain glycosaminoglycans, including dermatan sulfate and heparin, the rate of inhibition of thrombin increases ~1000-fold (6), while inhibition of chymotrypsin is unaffected (3). Current evidence suggests that binding of the glycosaminoglycan induces a conformational change in HCII that allows its N-terminal acidic domain to interact with anion-binding exosite I of thrombin (reviewed in Ref. 7). This interaction apparently facilitates proteolytic attack by thrombin at the reactive site of HCII.

We previously found that mutation of leucine 444 to arginine (L444R) at the P1 position of the reactive site of recombinant HCII (rHCII) increases the rate of inhibition of thrombin ~100-fold in the absence of a glycosaminoglycan (4). Furthermore, the L444R mutation abolishes the ability of rHCII to inhibit chymotrypsin. In the current study, we demonstrate differences in the stability of the thrombin–rHCII and thrombin–rHCII(L444R) complexes. The thrombin–rHCII(L444R) complex is much less stable in the presence of heparin than in the presence of dermatan sulfate and dissociates to yield active thrombin and a proteolytically cleaved form of rHCII(L444R). By contrast, the complex of thrombin with native HCII is relatively stable in the presence of either heparin or dermatan sulfate. Our data indicate that the P1 residue not only determines the protease specificity of HCII, as reflected by the relative rates of inhibition of thrombin and chymotrypsin, but also affects the stability of the resulting complex.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human α-thrombin was prepared as described previously (8) or purchased from Hematologic Technologies (Essex Junction, VT). Thrombin was labeled with Na$^{125}$I (ICN Biomedicals, Costa Mesa, CA) by the chloramine-T method (8) to attain a specific radioactivity of ~5.0 × 10$^4$ cpm/pmol. Active site-titrated human α-thrombin provided by Dr. Steven T. Olson (University of Illinois, Chicago, IL) was used to determine the stoichiometry of inhibition by rHCII. Bovine lung heparin was purchased from The Upjohn Co. Porcine skin dermatan sulfate was purchased from Sigma and treated with nitrous acid to degrade contaminating heparin or heparan sulfate (6).

Other materials were obtained from the following sources: isopropyl-1-thio-β-D-galactopyranoside, deoxyriboonuclease-I, 2-amino-2-methyl-1,3-propanediol (ammediol), and hirudin C-terminal peptide (residues 54–65, O-sulfated at Tyr-63, catalog no. H-6894) from Sigma; hexadimethrine bromide (Polybrene) from Aldrich; polyethylene glycol 8000 (PEG) from Union Carbo (Danbury, CT); tosyl-Gly-Pro-Arg-p-nitroanilide (Chromozym TH) from Boehringer Mannheim; H-α-Phe-pipocetyl-Arg-p-nitroanilide (S-2238) from Chromogenex AB (Mölndal, Sweden); heparin-Sepharose CL-6B, Mono Q, and Mono S columns from Pharmacia Biotech Inc.; restriction enzymes from New England Biolabs; and medium for high density bacterial culture from BLO 101 (Vista, CA).

**Expression and Purification of rHCII—**The full-length cDNA for native HCII was previously ligated into the pMON-5840 vector, and casserole mutagenesis was employed to construct the L444R mutation (4). For the current study, the cDNAs were removed from pMON-5840 and inserted between the NcoI and BamHI sites of the pET-3d expression vector (Novagen, Madison, WI). The mutations and ligation sites were verified by dideoxynucleotide sequencing (9).
Plasmid vectors were electroporated into Escherichia coli BL21(DE3)pLysS cells for expression. The cells were grown to an optical density (600 nm) of 5–6 in a BioFlo III high density fermentor (New Brunswick Scientific, Edison, NJ) at 37 °C and then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. The cells were harvested and lysed at 4 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and 2 mM EDTA. The lysate was treated with 10 μg/ml deoxyribonuclease-1 and 10 mM MnCl₂, and cellular debris was removed by centrifugation. rHCII was purified to homogeneity from the lysate by chromatography on heparin-Sepharose CL6B, Mono Q, and Mono S columns. HCII activity in the column fractions was determined in a thrombin inhibition assay in the presence of dermatan sulfate (4). The concentration of purified rHCII was determined by absorbance at 280 nm using the extinction coefficient determined for plasma HCII (1.17 ml/mg cm⁻¹) (10).

**Inhibition of Thrombin by rHCII**—Purified rHCII (native or L444R) was incubated with thrombin with or without a glycosaminoglycan at room temperature in 10 mM Tris-HCl, 150 mM NaCl, 1 mM PEG, pH 7.5 (TS/PEG buffer). At specified times ranging from 0.1 to 4500 min, 100 μl of the reaction mixture was mixed with 20 μM S-2238 for thrombin is 1.5 μM (12). A plot of \( \ln \left( \frac{[S]}{[I_0]} \right) \) versus time \( t \) according to Equation 1.

\[
\ln \left( \frac{[S]}{[I_0]} \right) = -k_{\text{obs}}t + \ln \frac{[S]}{[I_0]}_0
\]

\( [S]_0 \) and \( [I]_0 \) are the initial concentrations of S-2238 and rHCII, respectively. The rate constant for dissociation of the thrombin-rHCII complex at time zero, and \( k_{\text{obs}} \) is the rate of change in absorbance per unit time.

The reaction mixture was then diluted 25-fold into 0.4 mM S-2238, and \( k_{\text{obs}} \) was determined by Equation 2.

\[
k = k_{\text{obs}}[I]_0
\]

**RESULTS**

**Heparin Facilitates Dissociation of the Thrombin-rHCII(L444R) Complex**—Previously, we followed the reaction of thrombin with rHCII(L444R) for short periods of time (generally ≤5 min) to determine the initial rate of inhibition (4). In the experiment in Fig. 1, we monitored the time course of inhibition of thrombin by rHCII(L444R) or native rHCII for 4500 min. Thrombin incubated for 4500 min in buffer alone remains fully active (open circles). As previously reported (4), thrombin is inhibited much faster by rHCII(L444R) than by native rHCII in the absence of a glycosaminoglycan (compare closed squares, panels A and B). In the presence of heparin (closed circles) or dermatan sulfate (open circles), however, thrombin is inhibited rapidly by both forms of rHCII. Rate constants for inhibition of thrombin by rHCII(L444R), native
rHCII, and plasma HCII are compared in Table I. After being completely inhibited by either native rHCII or rHCII(L444R), thrombin activity remains undetectable for \(\geq4500\) min in the presence of dermatan sulfate. In the presence of heparin, however, a significant fraction of the thrombin activity inhibited by rHCII(L444R) reappears after a brief lag phase (Fig. 1, panel B, closed circles). Under the conditions of this experiment, thrombin activity reaches a plateau equal to \(50\%\) of its initial value at \(60\) min. By contrast, thrombin remains completely inhibited by native rHCII for \(4500\) min in the presence of heparin (Fig. 1, panel A, closed circles). Fig. 2 indicates the plateau level of thrombin released from rHCII(L444R) after \(720\) min in the presence of various concentrations of heparin or dermatan sulfate. Maximal dissociation of the thrombin-rHCII(L444R) complex occurs in the presence of low concentrations of heparin (\(0.2\ \mu\text{g/ml}\)), whereas no release of thrombin is observed at dermatan sulfate concentrations as high as 10 mg/ml. Dissociation of thrombin from rHCII(L444R), but not from native rHCII, also occurs at very high ionic strength (NaCl concentrations \(1.25\ M\) (data not shown), a phenomenon similar to that reported by Cooperman et al. (16) for the chymotrypsin-\(\alpha\)-antichymotrypsin complex.

The experiments in Figs. 1 and 2 were performed with a 35–40-fold molar excess of rHCII(L444R) with respect to thrombin. When higher concentrations of rHCII(L444R) are incubated with thrombin in the presence of heparin, the lag phase increases and the plateau of thrombin activity decreases (Fig. 3). Conversely, when lower concentrations of rHCII(L444R) are used, the initial inhibition of thrombin is incomplete, the lag phase disappears, and the plateau increases.

Stoichiometry of the Thrombin-rHCII(L444R) Reaction—It is apparent from the data in Fig. 3 that the stoichiometry of inhibition of thrombin by rHCII(L444R) in the presence of heparin is \(18:1\) (inhibitor:protease). To determine the stoichiometry more precisely, we incubated various amounts of native rHCII or rHCII(L444R) with thrombin for \(1\) min in the presence of heparin or dermatan sulfate and then measured the residual thrombin activity. Under each set of conditions, the stoichiometry was determined from a plot of thrombin activity versus rHCII concentration by extrapolation to zero thrombin activity (Table II). For both native rHCII and rHCII(L444R), the stoichiometry observed in the presence of heparin is \(2–3\) times higher than that observed in the presence of dermatan sulfate. Although the apparent stoichiometry with rHCII(L444R) in the presence of heparin is very high (i.e. \(24:1\)), this value may be overestimated somewhat because dissociation of a portion of the thrombin-rHCII(L444R) complexes may occur during the 1-min incubation (Fig. 3).

A stoichiometry of inhibition \(>1:1\) may reflect, in part, the presence of inactive protein in the rHCII preparation. However, quantitative cleavage of rHCII(L444R) by thrombin during the lag phase of the reaction shown in Fig. 4 (panel A) suggests that virtually \(100\%\) of the inhibitor is active.

### Table I

| Inhibitor | No GAG | + Dermatan sulfate | + Heparin |
|-----------|--------|--------------------|-----------|
| Plasma HCII | \(2.6 \times 10^4\) | \(6.3 \times 10^7\) | \(6.3 \times 10^7\) |
| rHCII(native) | \(1.9 \times 10^4\) | \(3.7 \times 10^7\) | \(4.3 \times 10^7\) |
| rHCII(L444R) | \(2.8 \times 10^6\) | \(1.1 \times 10^8\) | \(1.4 \times 10^8\) |

a GAG, glycosaminoglycan.

b Rate constants were determined as described under “Experimental Procedures.”

c Data are from Van Deerlin and Tollefsen (18).

### Table II

| Inhibitor | + Dermatan sulfate | + Heparin |
|-----------|--------------------|-----------|
| rHCII(native) | 3.8 | 7.0 |
| rHCII(L444R) | 8.2 | 23.5 |

Cleavage of Excess rHCII(L444R) during the Lag Phase—When rHCII(L444R) is incubated with thrombin in the presence of heparin for \(960\) min (during which time \(50\%\) of the thrombin dissociates from the complex) and then fresh rHCII(L444R) is added, all of the thrombin activity is again inhibited rapidly (data not shown). This result eliminates the possibility that dissociation yields an altered form of thrombin that cannot be inhibited by rHCII(L444R) and, furthermore, suggests that no active inhibitor is present in the incubation after the thrombin activity has reached its plateau level.

Fig. 4 (panel A) shows an SDS-PAGE analysis of rHCII(L444R) incubated with thrombin for \(0.2–180\) min in the presence of 1.25 MNaCl.
presence of heparin. Progressive conversion of excess rHCII(L444R) to a lower molecular weight form occurs during the 20-min lag phase, when thrombin activity is undetectable. As the thrombin activity approaches its plateau level between 60 and 120 min, intact rHCII(L444R) is no longer present. By contrast, only about 40% of the rHCII(L444R) is cleaved at 120 min in the presence of dermatan sulfate, and the amount of cleavage does not increase significantly thereafter (Fig. 4, panel B). The change in the molecular weight of rHCII(L444R) from -58,000 to -54,000 is consistent with proteolytic cleavage at the reactive site, which releases a peptide of 36 amino acids (17). Immunoblots probed with an antibody raised against the C-terminal decapeptide of HClII (residues 471–480) (18) indicate that this epitope is absent from the 54,000 molecular weight peptide, as expected for cleavage at the reactive site (data not shown). These results indicate that the thrombin-rHCII(L444R) complex dissociates in the presence of heparin to yield active thrombin and an inactive, proteolytically cleaved form of the inhibitor. Since thrombin released from the complex can react rapidly with intact inhibitor molecules as long as they are present in the incubation, thrombin activity remains undetectable during the lag phase of the reaction.

Conversion of Thrombin-rHCII(L444R) to a Non-dissociable Form—When thrombin is incubated with rHCII(L444R) for 17 min in the presence of heparin as in Fig. 1 (panel B) and then excess Polybrene (100 μg/ml) is added to neutralize the heparin, no release of thrombin activity occurs (data not shown). Therefore, thrombin-rHCII(L444R) complexes formed in the presence of heparin are stable if the heparin is neutralized soon after formation of the complex. Conversely, when heparin is added 17 min after initiation of the thrombin-rHCII(L444R) reaction, ~50% of the thrombin eventually dissociates from the inhibitor (Fig. 5, open squares). If heparin is added at progressively later times, however, the plateau decreases. After a 630-min preincubation in the absence of heparin, little or no thrombin activity is released from rHCII(L444R) by the subsequent addition of heparin. These results indicate that the thrombin-rHCII(L444R) complex slowly converts from a form that is dissociable by heparin to one that is not.

Conversion of the thrombin-rHCII(L444R) complex to the non-dissociable form can be prevented by a peptide that corresponds to the C-terminal portion of hirudin (residues 54–65), which binds to anion-binding exosite I of thrombin (19). As shown in Fig. 6 (closed circles), the thrombin-rHCII(L444R) complex dissociates completely in the presence of heparin when the hirudin peptide is also present throughout the incubation. The peptide does not cause dissociation of the complex in the absence of heparin (data not shown).

**Fig. 4. Cleavage of rHCII(L444R) during the lag phase of inhibition.** Thrombin (12 nM) was incubated with 378 nM rHCII(L444R) in the presence of 50 μg/ml heparin (panel A) or dermatan sulfate (panel B). At the times indicated, samples were assayed for thrombin activity and analyzed by SDS-PAGE on a 7.5% acrylamide gel stained with Coomassie Blue. The upper and lower bands on the gel have molecular weights of ~58,000 and ~54,000, respectively. The percentage of intact rHCII(L444R) (~57,58,000) at each time point is indicated (○).

**Fig. 5. Conversion of the thrombin-rHCII(L444R) complex to a non-dissociable form.** Thrombin (10 nM) was incubated with 378 nM rHCII(L444R) for 17 min (○), 30 min (■), 90 min (○), 180 min (●), or 630 min (△) before the addition of heparin (50 μg/ml final concentration). Each incubation was allowed to proceed for a total of 1500 min. Thrombin activity was determined in samples removed at the indicated times. In a control experiment, thrombin was incubated with rHCII(L444R) for 1500 min in the absence of heparin (▲).

**Fig. 6. Effect of hirudin C-terminal peptide on dissociation of the thrombin-rHCII(L444R) complex.** Incubations contained 10 nM thrombin, 480 nM rHCII(L444R), and 50 μg/ml heparin with (○) or without (△) 10 μM hirudin C-terminal peptide (residues 54–65). Incubations were initiated by addition of thrombin. Samples were removed at the indicated times and assayed for thrombin activity. Thrombin was incubated with buffer alone as a control (□).

**Kinetics of Dissociation of Thrombin-rHCII Complexes—**We determined rate constants (k−1) for dissociation of thrombin-rHCII(L444R) and thrombin-rHCII(native) complexes at various times of incubation in the absence or presence of a glycosaminoglycan. For these determinations, reaction mixtures containing the thrombin-rHCII complex were diluted into an ~105-fold molar excess of the chromogenic substrate S-2238. Dissociation of thrombin from the complex, indicated by a progressive increase in the rate of substrate hydrolysis, followed the first-order kinetic model described under “Experimental Procedures.” In the absence of a glycosaminoglycan, the
rate of dissociation of thrombin-rHCII(L444R) decreases with time (Fig. 7), corresponding with formation of the non-dissociable form of the complex. Rate constants ($k_{-1}$) calculated from the data are given in Table III. Rate constants for dissociation of thrombin-rHCII(native) in the absence of a glycosaminoglycan can also decrease with time and are similar to those of thrombin-rHCII(L444R). Both thrombin-rHCII complexes dissociate faster in the presence of heparin and somewhat slower in the presence of dermatan sulfate (Table III). However, heparin increases the rate constant for dissociation of thrombin-rHCII(L444R) to a much greater degree than that of thrombin-rHCII(native).

Stability of Thrombin-rHCII(L444R) Complexes in SDS—Serpin-protease complexes usually remain associated after denaturation in SDS (1). As shown in Fig. 8 (panel A), native rHCII rapidly forms an SDS-stable complex with $^{125}$I-thrombin in the presence of dermatan sulfate or heparin. In the absence of a glycosaminoglycan, the SDS-stable complex forms more slowly (the percentage of uncomplexed $^{125}$I-thrombin, determined by densitometry of the autoradiograph, is indicated by the open column below each lane). A small amount of radioactive material is also present on the gel between the free $^{125}$I-thrombin and complex bands and may represent proteolytically degraded complexes. Fig. 8 (panel B) shows the results of parallel incubations performed with rHCII(L444R). SDS-stable complexes form more rapidly in comparison with native rHCII in the absence of a glycosaminoglycan but less rapidly in the presence of dermatan sulfate or heparin.

In these experiments, there is an obvious discrepancy between the amount of residual thrombin activity, determined by hydrolysis of Chromozym TH (closed columns), and the amount of uncomplexed $^{125}$I-thrombin (open columns) detected in the gel. This discrepancy is most pronounced at the early time points with rHCII(L444R) (panel B, lanes 2, 5, and 8). A similar discrepancy is apparent in experiments with native rHCII in the absence of a glycosaminoglycan (panel A, lanes 2–4). These results indicate that inhibition of thrombin by either native rHCII or rHCII(L444R) may precede formation of an SDS-stable complex.

**DISCUSSION**

A general mechanism that describes the interactions of a protease with a serpin is shown in Fig. 9. The protease (E) and serpin (I) initially form a reversible Michaelis complex (E-I), which rapidly converts to the intermediate E-I*. E-I* probably represents a tetrahedral or acyl adduct involving the catalytic serine hydroxyl group of the protease and the carbonyl group of the P1 amino acid residue of the serpin (20, 21). Formation of E-I may be followed by a conformational change in the serpin, in which the reactive site loop containing the P1 residue becomes partially inserted into $\beta$-sheet A to form a kinetically stable complex (E-I*) (reviewed in Ref. 1). Alternatively, E-I may dissociate to yield the free protease (E) and a modified inhibitor (I*M) that has been cleaved proteolytically at the P1-P1' peptide bond. Rapid partitioning of the intermediate E-I along pathways leading to E-I* (inhibition pathway) or E + I*M (substrate pathway) determines the stoichiometry of inhibition (22). In general, E-I* is stable for many hours and can be considered the final product of the inhibition pathway. In some cases, however, E-I* may dissociate slowly to yield E + I*M (13, 23) or undergo further modification to yield a non-dissociable or "locked" form of the complex (E-I***) (16).

Mutation of leucine 444 to arginine at the P1 position of rHCII appears to affect several steps in this mechanism. 1) The L444R mutation alters the protease specificity of rHCII as reflected in the rates of inhibition of thrombin and chymotrypsin in the presence of a glycosaminoglycan. Native rHCII inhibits chymotrypsin more rapidly than thrombin (4). The L444R mutation increases the rate of inhibition of thrombin ~100-fold (Table I) while virtually abolishing inhibition of chymotrypsin. These results are consistent with the observation that the reactive site peptide bond (P1-P1') of a serpin generally mimics the substrate specificity of the target protease. Since thrombin preferentially attacks Arg-X peptide bonds, the presence of arginine at the P1 position of HCII probably increases the rate of formation of the Michaelis complex (E-I*), which is important in activating the inhibitory reactions.
Dissociation of the Thrombin-rHCII(L444R) Complex

Fig. 8. Formation of SDS-stable 125I-thrombin-rHCII(L444R) complexes. Incubations contained 7 nM thrombin (125I-labeled or unlabeled), 260 nM rHCII(native or L444R) and no glycosaminoglycan (−GAG), 50 μg/ml dermatan sulfate (+DS), or 50 μg/ml heparin (+Hep). After 5, 30, or 1200 min, samples from incubations containing unlabeled thrombin were assayed for thrombin activity by hydrolysis of Chromozym TH. Samples from incubations containing 125I-thrombin were heated for 4 min at 100 °C and then subjected to SDS-PAGE. Autoradiographs of the gels are shown in the upper panels. In the lower panels, thrombin activity (closed columns) is compared with the amount of uncleaved 125I-thrombin determined by densitometry of the autoradiograph (open columns); the results are expressed as percentages of control incubations containing only thrombin (Thr only).

Fig. 9. Proposed mechanism of action of a serpin. The serpin (I) reacts with a protease (E) to form a reversible Michaelis complex (E·I). E·I is rapidly converted to the intermediate E·I*, which then proceeds along either the Inhibition Pathway, yielding a kinetically stable complex (E·I**), or the Substrate Pathway, yielding a proteolytically modified form of the inhibitor (I_M) plus free protease (E). E·I** may dissociate slowly to yield E + I_M, or convert to a form in which the protease is irreversibly inhibited (E·I***).

Substrate pathways (reactions 3 and 4 in Fig. 9) occur rapidly (<1 min). Thus, the relatively slow release of thrombin cannot be explained by heparin favoring reaction 4 (E·I → E + I_M), since this would simply increase the stoichiometry of inhibition observed at short time points. When the ratio of rHCII(L444R) to thrombin exceeds the stoichiometry of inhibition (~24:1 in the presence of heparin), there is a lag phase during which thrombin activity is undetectable and the excess inhibitor is being cleaved to I_M (Fig. 4). Thrombin released from E·I* appears to be inhibited rapidly as long as intact rHCII(L444R) molecules remain present in the incubation. The net effect is that accelerated dissociation of the thrombin-rHCII(L444R) complex in the presence of heparin leads to progressive degradation of the excess inhibitor via reactions 4 and 5 (Fig. 9).

The experiment in Fig. 4 also provides evidence for turnover of the thrombin-rHCII(L444R) complex in the presence of dermatan sulfate. Approximately 15% of the rHCII(L444R) is cleaved after a 5-min incubation with thrombin and dermatan sulfate, in reasonable agreement with the value predicted from the stoichiometry of inhibition (7.2 × 12/378 = 3%). Further cleavage of rHCII(L444R) occurs over 180 min, consistent with the slow rate of dissociation documented in Table III. The amount of cleaved rHCII(L444R) appears to level off at ~40% as thrombin becomes trapped in the non-dissociable form of the complex. Therefore, under the conditions of this experiment, no thrombin activity is released even after much longer times of incubation.

When rates of dissociation are determined in the presence of excess S-2238, which prevents de novo formation of thrombin-rHCII complexes, we find that thrombin-rHCII(L444R) and thrombin-rHCII(native) dissociate with similar half-lives in the range of 1300–2000 min (t_{1/2} = 690/k_{-1}) in the absence of a glycosaminoglycan (Table III). These rates are somewhat faster than that of the thrombin-antithrombin complex (t_{1/2} = 8000 min) (13). Heparin increases the rate of dissociation of thrombin-rHCII(L444R) approximately 60-fold (t_{1/2} = 21 min) but has much less of an effect on the rate of dissociation of thrombin-rHCII(native) (t_{1/2} = 300 min). Therefore, the L444R mutation does not affect the inherent stability of thrombin-rHCII(L444R) but somehow enables heparin to destabilize the complex.

4) The thrombin-rHCII(L444R) complex converts to a non-dissociable form (designated E·I*** in Fig. 9) in a time-dependent manner. Thus, the plateau of thrombin activity decreases by ~50% when heparin is present throughout the incubation to ~2% when heparin is added 630 min after formation of the complex (Fig. 5). Because conversion of E·I* to E·I*** (reaction 6 in Fig. 9) competes with heparin-induced dissociation of E·I* (reaction 5 in Fig. 9), ~100% of the thrombin is ultimately released.

The hirudin C-terminal peptide prevents conversion of thrombin-rHCII(L444R) to the non-dissociable form (Fig. 6). This peptide binds to anion-binding exosite I of thrombin and inhibits proteolysis of certain macromolecular substrates (e.g. fibrinogen) (19). Although the hirudin peptide markedly decreases the rate of inhibition of thrombin by native rHCII in the presence of a glycosaminoglycan (18), it has little effect on the initial rate of inhibition of thrombin by rHCII(L444R) (Fig. 6). The effect of the hirudin peptide was consistent with a proteolytic mechanism in which E·I* is converted to E·I*** by a trace amount of the free enzyme, as suggested by Cooperman et al. (16) for the chymotrypsin-α₂-antichymotrypsin complex. Alternatively, the hirudin peptide could bind directly to thrombin-rHCII(L444R) and disrupt interactions between the N-terminal acidic domain of rHCII and anion-binding exosite I of thrombin that may serve to stabilize the complex.

Conversion to the non-dissociable form parallels the progressive decrease in the rate constant for dissociation (k_{-1}) of thrombin-rHCII(L444R) observed in the absence of a glycosaminoglycan (Table III). Similarly, the k_{-1} of thrombin-rHCII(native) decreases with time, suggesting that this complex also converts to a non-dissociable form. The data in Table III suggest that reaction 6 (Fig. 9) is relatively slow with a t_{1/2} in the range of 30–60 min. If the reaction is not first-order as implied in Fig. 9, but instead depends on the concentration of free...
Dissociation of the Thrombin-rHCII(L444R) Complex

proteinase as suggested by Cooperman et al. (16), then reaction 6 could occur much faster during the initial phase of the reaction before the protease has been inhibited completely. This could explain why <100% of the thrombin is released from thrombin-rHCII(L444R) even if heparin is present throughout the incubation (e.g. Figs. 1 and 3).

5) The inhibitory pathway of a serpin-protease reaction is generally accompanied by formation of an SDS-stable complex indicative of a covalent linkage between the two proteins. By generally accompanied by formation of an SDS-stable complex incubation (rHCII(L444R) even if heparin is present throughout the explain why

could occur much faster during the initial phase of the reaction

binding of heparin to specific amino acid residues on one or both of these proteins. Investigation of thrombin-rHCII(L444R) has revealed aspects of serpin biochemistry, such as induced dissociation of the complex and conversion to a “locked” form, that are not widely recognized. These reactions may exemplify important mechanisms by which the activities of serpins can be regulated.

Acknowledgment—We thank Steve Olson for advice during the course of this investigation and for the gift of active site-titrated thrombin.

REFERENCES

1. Gettins, P. G. W., Patston, P. A., and Olson, S. T. (1996) Serpins: Structure, Function and Biology, R. G. Landes Company, Austin, TX
2. Parker, K. A., and Tollefsen, D. M. (1985) J. Biol. Chem. 260, 3501–3505
3. Church, F. C., Noyes, C. M., and Griffith, M. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6431–6434
4. Derechin, V. M., Blinder, M. A., and Tollefsen, D. M. (1990) J. Biol. Chem. 265, 5623–5628
5. Griffith, M. J., Noyes, C. M., Tyndall, J. A., and Church, F. C. (1985) Biochemistry 24, 6777–6782
6. Tollefsen, D. M., Pestka, C. A., and Menafo, W. J. (1983) J. Biol. Chem. 258, 6715–6716
7. Tollefsen, D. M. (1995) Thromb. Haemost. 74, 1209–1214
8. Tollefsen, D. M., and Blank, M. K. (1981) J. Clin. Invest. 68, 589–596
9. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
10. Tollefsen, D. M., Majerius, D. W., and Blank, M. K. (1982) J. Biol. Chem. 257, 2162–2169
11. Olson, S. T., Bjork., I., and Shore, J. D. (1993) Methods Enzymol. 222, 525–559
12. Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., and Jackson, C. M. (1983) Biochim. Biophys. Acta 742, 539–557
13. Jestly, J. (1979) J. Biol. Chem. 254, 10044–10050
14. Mast, A. E., Enghild, J. J., and Salvesen, G. (1992) Biochemistry 31, 2720–2728
15. Lennmari, U. K. (1970) Nature 227, 680–685
16. Cooperman, B. S., Stavridi, E., Nickbarg, E., Rescorla, E., Schechter, N. M., and Rubin, H. (1990) J. Biol. Chem. 265, 23616–23625
17. Blinder, M. A., Marasa, J. C., Reynolds, C. H., Deaven, L. L., and Tollefsen, D. M. (1998) Biochemistry 27, 752–759
18. Van Deurzen, V. M. D., and Tollefsen, D. M. (1991) J. Biol. Chem. 266, 20223–20231
19. Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton, J. W., II. (1990) Science 249, 277–280
20. Matheson, N. R., van Halbeek, H., and Travis, J. (1991) J. Biol. Chem. 266, 13489–13491
21. Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Krasserman, J. O., and Shore, J. D. (1990) J. Biol. Chem. 270, 25309–25312
22. Olson, S. T. (1985) J. Biol. Chem. 260, 10153–10160
23. Danielsson, Å., and Bjork, I. (1983) Biochem. J. 213, 345–353