Mutagenesis of Thrombin Selectively Modulates Inhibition by Serpins
Heparin Cofactor II and Antithrombin III

INTERACTION WITH THE ANION-BINDING EXOSITE DETERMINES HEPARIN COFACTOR II SPECIFICITY

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Thrombin is a multifunctional serine protease that plays a critical role in hemostasis. Thrombin is inhibited by the serpins antithrombin III and heparin cofactor II in a reaction that is dramatically accelerated by glycosaminoglycans. The structural basis of the interaction with these inhibitors was investigated by introducing single amino acid substitutions into the anion-binding exosite (R68E, R70E) and unique insertion loops (K52E, K154A) of thrombin. The rate of inhibition of these recombinant thrombins by antithrombin III and heparin cofactor II was determined in the absence and presence of glycosaminoglycans. The second order rate constant (k2) for inhibition by antithrombin III without heparin was 3.7 x 10^9 M^-1 min^-1 for wild-type thrombin; rates for the mutant thrombins varied less than 2-fold. For inhibition by antithrombin III with heparin, the rate constant was 4.5 x 10^9 M^-1 min^-1 for wild-type thrombin with no significant differences between any of the recombinant thrombins. In contrast, the rate constant for inhibition by heparin cofactor II without glycosaminoglycan was 4.3 x 10^9 M^-1 min^-1 for wild-type thrombin; rates were 10-fold slower for thrombin K52E and 2- to 3-fold slower for thrombins R68E and R70E. The rate constants for inhibition of wild-type thrombin by HCII in the presence of heparin or dermatan sulfate were 9.2 x 10^9 M^-1 min^-1 and 9.0 x 10^9 M^-1 min^-1, respectively. Compared to wild-type thrombin, the rate of inhibition by HCII with glycosaminoglycan was 5- to 15-fold slower for thrombins K52E and R70E and 50- to over 100-fold slower for thrombin R68E. Thrombin K154A was inhibited by heparin cofactor II with rates similar to wild-type thrombin in all assays. These results suggest that heparin cofactor II interacts with residues Lys-52 in the proposed S1' subsite and with residues Arg-68 and Arg-70 in the anion-binding exosite of thrombin, and that these interactions contribute to the molecular basis of heparin cofactor II specificity for thrombin.

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† The abbreviations used are: ATIII, antithrombin III; HCII, heparin cofactor II; PAGE, polyacrylamide gel electrophoresis; PEG-8000, polyethylene glycol, average M, = 8000.
EXPERIMENTAL PROCEDURES

Materials—Human plasma heparin cofactor II and antithrombin III were purified by heparin-Sepharose chromatography as previously described (27). Human plasma prothrombin was obtained from Joseph Miletich and George J. Broze, Jr. (Washington University, St. Louis). Porcine intestinal mucosal heparin was purchased from Lyphomed, Inc. (Rosemont, IL). Polyethylene glycol (PEG-8000), vitamin K1, bovine insulin, human transferrin, bovine fetuin, Ox yrurus scutellatus venom, rabbit brain cephalin, and bovine mucosal derman- tan sulfate were purchased from Sigma. Dermatan sulfate was treated with nuclease to remove contaminating heparin (28). Chromogenic substrate S-2238 (H-D-Phe-picolyl-PArg-g-nitroanilide) was purchased from KabiVitrum. Cytodec-2, cross-linked dextran beads for cell culture, were purchased from Pharmacia LKB Biotechnology Inc. 

Plasmid Constructs—Plasmid pCMVPT, containing a full-length human prothrombin cDNA inserted into the PCMV expression vector, and recombinant mutant prothrombin plasmids pKM52E, pR66E, and pR70E were described previously (8). Plasmid pK154A, in which the codon AAG for Lys-154 of the scutellatus venom, rabbit brain cephalin, and bovine mucosal derman- tan (24). Deletion of this acidic domain from recombinant HCII mark- edly reduces the acceleration of thrombin inhibition by glycosaminoglycan (26). Thus, the interaction of the acidic do- main of HCII with the anion-binding exosite may contribute to the specificity of this inhibitor for thrombin.

The aim of the present study was to identify amino acid residues of thrombin that are important for interaction with the inhibitors ATIII and HCII. Single amino acid substitutions were introduced into surface residues of thrombin that are unique compared with homologous serine proteases. Mu- tations in the proposed S1’ subsite (Lys-52) and the anion-binding exosite (Arg-68 and Arg-70) selectively decreased the rate of thrombin inhibition by HCII, especially in the presence of glycosaminoglycan. This suggests that interaction of HCII with these unique surface residues contributes to the molec- ular basis of its specificity for thrombin.

Glycosaminoglycan-independent Thrombin Inhibition—Inhibition assays were performed under pseudo-first order conditions in the presence of 150 nM ATIII or 410 nM HCII, 15 to 30 nm thrombin, 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 0.1% PEG-8000 at room temperature. Inhibition reactions for ATIII in the absence of heparin also contained 4.5 μg/ml Polybrene to neutralize any traces of heparin in the ATIII preparation. The inhibition reaction was initiated by the addition of thrombin and sampled at appropriate time points to assay remaining thrombin activity by the rate of S-2238 hydrolysis. Inhibition by ATIII or HCII was quenched by adding a 50-μl sample to 250 μl of 300 μM S-2238, 5.4 μg/ml Polybrene, and 0.12 kallikrein- inactivating units/ml aprotinin. The rate of S-2238 hydrolysis was determined from the rate of change of absorbance at 450 nm over 120 s on a Beckman DU-7 spectrophotometer. Efficacy of inhibition quenching was confirmed by observing: 1) a constant rate of thrombin activity for at least 5 min following addition to the quenching solution; and 2) equivalent thrombin activity both in control assays without serum and in assays where the thrombin was added to quenching solution containing serpin.

Rate constants for the glycosaminoglycan-independent inhibition were obtained by fitting the exponential function of Equation 1 to the data by nonlinear least squares regression using the program KINSIM (39) and FITSIM (40) on a Digital Electronics Corporation MicroVAX II. KINSIM allows simulation of kinetic mechanisms by numerical integration. FITSIM was used with the Marquardt algorithm for the non-linear least squares regression analysis.

Glycosaminoglycan-dependent Thrombin Inhibition—Inhibition as- says in the presence of glycosaminoglycan were performed with 150 nM ATIII or HCl under the same conditions used for the serpin inhibition assays: 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 0.1% PEG-8000 at room temperature. For each recombinant thrombin, Kd was determined from full progress curves obtained with a dual-beam Cary 3 spectrophotometer. Substrate concentrations of 2 and 20 μM S-2238 were used with signal averaging times of 0.4 and 1.0 s, respectively. The thrombin concentration was between 1 and 5 nM. The progress curves were fit to the kinetic scheme:

\[ E + S \rightleftharpoons ES \rightarrow EP \rightleftharpoons E + P \]

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Purity of prothrombins was confirmed by 10% SDS-PAGE (36) and silver staining (37).

Prothrombins were activated to thrombin by O. scutellatus venom in the presence of phospholipid and calcium chloride (38). Prothrom- bins were diluted to approximately 30 μg/ml with 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.1% PEG-8000. The diluted prothrombins were then incubated at 37 °C for 18 min after the addition of 0.01 volumes of 10 mg/ml O. scutellatus venom, 0.02 volume of rabbit brain cephalin, and CaCl2 to a final concentration of 5 mM. Complete activ- ation to thrombin was confirmed by Western blotting of 10% SDS- PAGE gels. Controls containing equivalent amounts of O. scutellatus venom without thrombin showed no significant background cleavage of S-2238 under the conditions employed for either Kd determinations or serpin inhibition assays.

Determination of Michaelis constant (Km) for S-2238—The value of Kd for the chromogenic substrate S-2238 was determined for plasma- derived thrombin and each recombinant thrombin under the same conditions used for the serpin inhibition assays: 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 0.1% PEG-8000 at room temperature. For each recombinant thrombin, Kd was determined from full progress curves obtained with a dual-beam Cary 3 spectrophotometer. Substrate concentrations of 2 and 20 μM S-2238 were used with signal averaging times of 0.4 and 1.0 s, respectively. The thrombin concentration was between 1 and 5 nM. The progress curves were fit to the kinetic scheme:

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Glycosaminoglycan-independent Thrombin Inhibition—Inhibition assays were performed under pseudo-first order conditions in the presence of 150 nM ATIII or 410 nM HCII, 15 to 30 nm thrombin, 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 0.1% PEG-8000 at room temperature. Inhibition reactions for ATIII in the absence of heparin also contained 4.5 μg/ml Polybrene to neutralize any traces of heparin in the ATIII preparation. The inhibition reaction was initiated by the addition of thrombin and sampled at appropriate time points to assay remaining thrombin activity by the rate of S-2238 hydrolysis. Inhibition by ATIII or HCII was quenched by adding a 50-μl sample to 250 μl of 300 μM S-2238, 5.4 μg/ml Polybrene, and 0.12 kallikrein- inactivating units/ml aprotinin. The rate of S-2238 hydrolysis was determined from the rate of change of absorbance at 450 nm over 120 s on a Beckman DU-7 spectrophotometer. Efficacy of inhibition quenching was confirmed by observing: 1) a constant rate of thrombin activity for at least 5 min following addition to the quenching solution; and 2) equivalent thrombin activity both in control assays without serpin and in assays where the thrombin was added to quenching solution containing serpin.

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Glycosaminoglycan-dependent Thrombin Inhibition—Inhibition as- says in the presence of glycosaminoglycan were performed with 150 nM ATIII or HCII under the same conditions as above but in the presence of competing substrate (S-2238). Inhibition by ATIII was assayed with either 1 unit/ml heparin or 100 μg/ml dermanatan sulfate. The glycosaminoglycan concentrations chosen gave maximal rates of thrombin inhibition (data not shown).

For the glycosaminoglycan-dependent reaction, the observed rate constant (k') was obtained by Equation 1 above. The k' was then corrected for substrate competition and divided by the inhibitor concentration to give the second order rate constant k'(41) as shown in Equation 2 where [S] represents the substrate concentration pres-
ent during the reaction and $k_r$ represents the Michaelis constant of the substrate for each thrombin.

$$k_r'(1 + [S]/K_m) = k_3[I]$$  \hspace{1cm} (Eq 2)

**HCII-Thrombin Complex Formation**—The time course of HCII-thrombin complex formation was followed at room temperature for 60 min in 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.1% PEG-8000 containing 25 nM HCII and approximately 15 nM recombinant thrombin. Thrombin was added last to initiate the reaction which was subsequently sampled at 1, 3, 30, and 60 min by removing 45 µl and mixing with 15 µl of SDS-PAGE loading buffer (4X) containing 2-mercaptoethanol. Samples were then heated at 90 °C for 3 min and analyzed by 10% SDS-PAGE. Proteins were electrotransferred to a nitrocellulose membrane, incubated with 5% BLOTTO (5% condensed milk, 0.05 M NaCl, 0.05 M Tris-HCl pH 7.4) for 2 h at room temperature, followed by incubation with 100 µg of polyclonal rabbit anti-HCII antibody (42) in 50 ml of 1% BLOTTO for 2 h at 37 °C. The nitrocellulose membrane was then washed with 1% BLOTTO and incubated with $3 	imes 10^6$ cpm of $^{125}$I-labeled goat anti-rabbit IgG in 1% BLOTTO for 2 h at 37 °C. The nitrocellulose membrane was again washed with 1% BLOTTO followed by phosphate-buffered saline and exposed to prefilmed film (Kodak X-OMAT AR) overnight.

**RESULTS**

Four single-amino acid substitutions were constructed in surface residues of the human thrombin B chain. Three of these change basic amino acid residues to acidic residues: K52E is in the 9-residue β-hairpin insertion loop that is proposed to be part of the S1' site (3), R68E and R70E are located in the anion-binding exosite (3–5). The fourth substitution, K154A, is located in a 5-residue insertion loop that contributes to the lower lip of the active site cleft (3). The residue corresponding to K154 of human thrombin is glutamic acid in bovine thrombin (43). Since bovine thrombin is known to be inhibited at rates similar to those for human thrombin by ATIII and HCII (44), substitution was made to a neutral amino acid to determine whether an uncharged residue would affect the interaction.

**Determination of $K_m$ Values for Recombinant Thrombins**—The value of $K_m$ for S-2238 was determined for plasma-derived thrombin and each recombinant thrombin (Table I). The results were quite similar for plasma-derived thrombin, wild-type thrombin, and each of the mutant thrombins, indicating that these mutations did not significantly perturb the active site. Similar results were reported previously under slightly different conditions for plasma-derived thrombin, wild-type thrombin, and each of the mutant thrombins (K52E, R68E, and R70E (8). The absence of significant differences in $K_m$ for S-2238 (Table I) indicates that the differences in observed glycosaminoglycan-dependent inhibition rates were not due to changes in the ability of the substrate S-2238 to compete for the active site.

**Inhibition of Thrombin by ATIII**—The rate of inhibition of plasma-derived and recombinant thrombins by ATIII was determined under pseudo-first order conditions (Fig. 1A). Wild-type thrombin was inhibited with a rate constant ($k_3$) of $3.7 	imes 10^4$ M$^{-1}$ min$^{-1}$ (Table I) which was similar to that of plasma-derived thrombin and consistent with previously reported values ($4.25 	imes 10^4$ M$^{-1}$ min$^{-1}$, $3.2 	imes 10^4$ M$^{-1}$ min$^{-1}$) (16, 44). Among the mutant thrombins, the largest difference observed was for thrombin K52E with a $k_3$ approximately one-half that of wild-type thrombin. The K52E mutation is located closest to the active site in the proposed S1' subsite. The rate of inhibition of the other mutant thrombins varied less than 2-fold from that of wild-type thrombin.

**Inhibition of Thrombin by ATIII plus Heparin**—The rate of inhibition of plasma-derived and recombinant thrombins by ATIII in the presence of heparin was determined by a substrate competition assay under pseudo-first order conditions (Fig. 1B). Inhibition of recombinant wild-type thrombin was accelerated over 1000-fold by heparin to a mean rate constant of $4.5 	imes 10^6$ M$^{-1}$ min$^{-1}$ (Table I). This rate of inhibition is similar to that of plasma-derived thrombin and consistent with previously reported values ($1.7 	imes 10^6$ M$^{-1}$ min$^{-1}$, $3 	imes 10^5$ M$^{-1}$ min$^{-1}$) (16). There were no significant differences among the $k_3$ values for the mutant thrombins (Table I), and all were inhibited at rates similar to that of wild-type thrombin. Thus, none of these mutations appeared to significantly affect the interaction of thrombin with ATIII in the presence of heparin.

**Inhibition of Thrombin by HCII**—The effect of mutations on the inhibition of thrombin by HCII was determined under pseudo-first order conditions (Fig. 2A). Wild-type thrombin was inhibited with a rate constant of $4.3 	imes 10^5$ M$^{-1}$ min$^{-1}$ which was similar to thrombin K154A and plasma-derived thrombin. These rates were consistent with previously reported values for the inhibition of plasma-derived thrombin by HCII ($6.0 	imes 10^4$ M$^{-1}$ min$^{-1}$, $5.5 	imes 10^4$ M$^{-1}$ min$^{-1}$) (44, 45). The exosite mutants R68E and R70E were inhibited at 2- to 3-fold slower rates while thrombin K52E was inhibited at a 10-fold slower rate than wild-type thrombin (Table I). In comparison to inhibition by ATIII, mutations in these unique surface residues of thrombin (K52, R68, and R70) appear to selectively affect thrombin interaction with HCII.

**Inhibition of Thrombin by HCII Plus Heparin**—The substrate competition assay was used to determine the effect of these mutations on the inhibition of thrombin by HCII in the presence of heparin (Fig. 2B). Inhibition of both wild-type

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**Table I**

| Thrombin | $K_m$ S-2238 (µM) | ATIII (>100) | ATIII + heparin (>100) | HCII (>100) | HCII + heparin (>100) | HCII + dermatan sulfate (>100) |
|----------|-----------------|-------------|----------------------|------------|----------------------|-----------------------------|
| pIIa     | 3.0 ± 0.3       | 3.5 ± 0.6   | 3.9 ± 0.5            | 4.0 ± 0.3  | 64.9 ± 14            | 63.1 ± 5.4                  |
| rIIa     | 3.1 ± 0.7       | 3.7 ± 0.3   | 4.5 ± 1.1            | 4.3 ± 0.9  | 92.0 ± 14            | 89.8 ± 28                   |
| K52E     | 3.8 ± 0.6       | 1.8 ± 0.7   | 5.4 ± 0.8            | 0.4 ± 0.2  | 8.7 ± 1.6            | 9.6 ± 1.0                   |
| R68E     | 3.5 ± 0.5       | 2.5 ± 0.5   | 4.4 ± 1.6            | 2.2 ± 0.3  | 1.7 ± 0.4            | 0.7 ± 0.2                   |
| R70E     | 3.3 ± 0.8       | 3.6 ± 0.5   | 4.6 ± 1.1            | 1.6 ± 0.3  | 16.6 ± 1.3           | 10.8 ± 4.9                  |
| K154A    | 2.7 ± 0.2       | 5.4 ± 0.4   | 5.8 ± 1.8            | 3.9 ± 0.3  | 70.1 ± 2.1           | 45.0 ± 3.2                  |

**Kinetic constants for plasma and recombinant thrombins**

$K_m$ values for the hydrolysis of S-2238 were determined from full progress curves (see "Experimental Procedures") and are expressed in the left hand column ± S.D. Rate constants ($k_3$) for inhibition by ATIII or HCII are shown on the right-hand side of the table for each condition described in the legends of Figs. 1 and 2. Data are expressed as the mean of three or more determinations ± S.D. pIIa represents plasma thrombin, rIIa represents wild-type thrombin.
Serpin Inhibition of Recombinant Thrombin

FIG. 1. Thrombin inhibition by ATIII. Representative inhibition curves are shown in the absence of glycosaminoglycan (panel A) or the presence of 1 unit/ml heparin (panel B). Thrombins were added at a final concentration of 15 to 30 nM to a reaction mixture containing 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% PEG-8000, and 150 nM ATIII. Polybrene (4.5 μg/ml) was present in the reaction without heparin, and 150 μM S-2238 was present in the heparin-dependent reaction. Reactions were sampled at the appropriate times, and the remaining thrombin activity was determined by the rate of S-2238 hydrolysis (see “Experimental Procedures”). Wild-type thrombin, ○; thrombin K52E, □; thrombin R68E, ■; thrombin R70E, △; thrombin K154A, ▲.

Thrombin and thrombin K154A by HCII was accelerated 18,000- to 20,000-fold by heparin. Wild-type thrombin exhibited a rate constant of 9.2 × 10^8 M⁻¹ min⁻¹, and a similar value was obtained for thrombin K154A. Plasma-derived thrombin was inhibited at a slightly slower rate than recombinant wild-type thrombin (Table I), but the rate constant obtained was similar to previously reported values (3.0 × 10^8 M⁻¹ min⁻¹, 3.8 × 10^8 M⁻¹ min⁻¹) (18, 27). The rate of inhibition of thrombin K52E by HCII was accelerated 16,000-fold by heparin, and it remained over 10-fold slower than wild-type thrombin. By comparison, the inhibition of thrombins R70E and R68E by HCII was accelerated by heparin only 10,000-fold and 1,000-fold, respectively. Rate constants were reduced by 6-fold for thrombin R70E and over 50-fold for R68E compared to wild-type thrombin (Table I).

Inhibition of Thrombin by HCII Plus Dermatan Sulfate—The results for inhibition of thrombin by HCII in the presence of dermatan sulfate (Fig. 2C) paralleled those obtained in the presence of heparin. Inhibition of wild-type thrombin by HCII was accelerated over 20,000-fold by dermatan sulfate. Wild-type thrombin exhibited a rate constant of 8.9 × 10^8 M⁻¹ min⁻¹; rate constants for thrombin K154A and plasma-derived thrombin were slightly slower but consistent with previously reported values for plasma-derived thrombin (6.3 × 10^8 M⁻¹ min⁻¹) (18). Relative to wild-type thrombin, rate constants were reduced over 100-fold for thrombin R68E, 8-fold for thrombin R70E, and nearly 10-fold for thrombin K52E. Acceleration of the inhibition by dermatan sulfate was again significantly less for thrombins R68E and R70E compared to other recombinant thrombins. The pronounced effect of these mutations on the inhibition rate in the presence of heparin

FIG. 2. Thrombin inhibition by HCII. Representative inhibition curves are shown in the absence of glycosaminoglycan (panel A), in the presence of 10 units/ml heparin (panel B), or in the presence of 100 μg/ml dermatan sulfate (panel C). Thrombins were added at a final concentration of 15 to 30 nM to a reaction mixture containing 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% PEG-8000, and 410 nM or 150 nM HCII in the absence or presence of glycosaminoglycan, respectively. In the glycosaminoglycan-dependent assays (panels B and C), 150 μM S-2238 was present. Reactions were sampled at the appropriate times, and the remaining thrombin activity was determined by the rate of S-2238 hydrolysis (see “Experimental Procedures”). Wild-type thrombin, ○; thrombin K52E, □; thrombin R68E, ■; thrombin R70E, △; thrombin K154A, ▲.
or dermatan sulfate suggests HCII has significant interactions with these surface residues on thrombin: Lys-52 in the active site cleft and Arg-68 and Arg-70 in the anion binding exosite. The interactions with Arg-68 and Arg-70 appear to be critical for accelerating the rate of HCII inhibition in the presence of glycosaminoglycan.

**HCII-Thrombin Complex Formation**—In principle, mutations in thrombin could slow the rate of HCII inhibition by enabling the mutant enzymes to utilize HCII mainly as a substrate rather than to form inactive HCII-thrombin complexes. To address this possibility, the time course of HCII-thrombin complex formation was monitored by Western blotting under conditions of slight inhibitor excess. Comparison of thrombins K52E and R68E to wild-type thrombin (Fig. 3) demonstrated a slightly slower rate of complex formation for thrombin K52E and markedly slower rate for thrombin R68E. This agrees with their respective $k_2$ values determined in the HCII inhibition assays. In addition, the cleaved form of HCII appeared rapidly during the course of complex formation for wild-type thrombin, but more slowly and simultaneously with complex formation for thrombin R68E. This is consistent with results reported for ATIII in the presence of heparin, where substrate cleavage of the inhibitor and complex formation result from a common intermediate (46). This result demonstrated that the slower rate of inhibition for thrombin R68E is not secondary to increased cleavage of the inhibitor and that the mutant enzyme is capable of forming stable complexes with HCII. Thrombins R70E and K154A (data not shown) similarly demonstrated simultaneous appearance of the HCII-thrombin complex and the cleaved form of HCII at rates proportional to their previously determined $k_2$ values. At early time points, thrombin K52E demonstrated a decreased proportion of cleaved HCII relative to complex formation. Western blotting with a polyclonal rabbit antibody against the COOH-terminal peptide of HCII also demonstrated slower cleavage of the COOH-terminal peptide by thrombin K52E (data not shown). This suggests that the K52E mutation affects the ability of thrombin to cleave HCII.

**DISCUSSION**

The main determinant of serpin target specificity is the P1 residue which reflects the substrate specificity of the inhibited protease (12). The P1 arginine of ATIII largely determines its specificity for a broad range of trypsin-like proteases including thrombin. In contrast, the P1 leucine of HCII is responsible for its ability to inhibit chymotrypsin but does not determine specificity for thrombin (47). Inhibition of thrombin by HCII must require additional structural interactions that explain this specificity. Van Deerlin and Tollefson (26) and Ragg et al. (25) have proposed that the amino-terminal acidic domain of HCII interacts with the anion-binding exosite of thrombin. In this model, glycosaminoglycan binds to HCII, displacing the amino-terminal acidic domain from an intramolecular binding site. This acidic domain can then bind to the exosite of thrombin (24) resulting in acceleration of the inhibition. Deletion of the amino-terminal acidic domain of recombinant HCII results in a markedly decreased ability of glycosaminoglycan to accelerate the inhibition rate (26). Based on this model, the interaction of thrombin and HCII should be adversely affected by mutations in the anion-binding exosite, whereas the interaction of ATIII with the active site should be unaffected.

Mutagenesis of single amino acids allowed the identification of specific thrombin residues that interacted with HCII but not with ATIII. These mutations were constructed either in insertion loops unique to thrombin or a secondary binding site for large substrates, the anion-binding exosite. This strategy avoids the large structural changes associated with proteolytically modified thrombin derivatives and provides a degree of precision that is difficult to achieve with chemical modification methods. The tertiary structure and catalytic mechanism of the mutant thrombins were inferred to be intact from the $K_w$ values obtained with the peptide substrate S-2238; all of these values were similar to those obtained for plasma-derived thrombin and wild-type recombinant thrombin. Wild-type recombinant thrombin possessed essentially normal activity toward macromolecular substrates or cofactors such as fibrinogen, thrombomodulin, protein C, platelets (8), and the serpins ATIII and HCII (Figs. 1 and 2). The selective effects of single-amino acid substitutions on the interaction of several mutant thrombins with specific ligands also provide evidence of intact tertiary structure (8).

In the absence of heparin, only small differences in the rate of inhibition by ATIII were seen between any of the mutant thrombins and wild-type thrombin. Thrombin K52E was inhibited most slowly, which is not unexpected since Lys-52 may be part of the S1' subsite (3) that interacts with the reactive loop of ATIII. The substitution K52E could potentially affect the initial binding of ATIII or subsequent formation of the stable enzyme-inhibitor complex. In the presence of heparin, none of these mutations significantly affected the rate of inhibition by ATIII. Thrombin K52E was inhibited at the same rate as wild-type thrombin, suggesting either that heparin accelerates the reaction efficiently to minimize any contribution by residue Lys-52 or that heparin induces a conformational change in thrombin making this residue unimportant for inhibition. The lack of effect of these thrombin mutations on the inhibition rate suggests that ATIII does not interact significantly with residue Lys-154, the anion-binding exosite residues Arg-68 and Arg-70, or residue Lys-52 (in the presence of heparin). The broad protease specificity of ATIII may result from the lack of interactions with unique surface residues of thrombin located on the carboxyl side of the scissile bond and instead may depend primarily on interaction with conserved regions of the trypsin-like proteases.

By site-directed mutagenesis of the P1' serine of ATIII, Stephens et al. (48) demonstrated that interaction with this residue is not critical for inhibition of thrombin. Depending on the specific substitution for the P1' serine of ATIII, the
rate of thrombin inhibition was minimally to markedly slowed. This effect appeared to correlate with the relative size and hydrophobicity of the P1' residue. In the presence of heparin, however, the differences in inhibition rate among the mutants were reduced or no longer apparent. These results demonstrate a permissive interaction of the S1' subsite of thrombin with the P1' residue of ATIII that has relatively less importance in the presence of heparin. This is consistent with the results obtained for thrombin K52E which contains a mutation in the proposed S1' subsite of thrombin.

In contrast, thrombin inhibition by HCII was significantly affected by several of these mutations. In the absence of glycosaminoglycan, modest reductions in the inhibition rate were observed for mutations in the anion-binding exosite (R68E and R70E), and a marked reduction in rate was observed for the mutation in the propose S1' subsite (K52E).

No significant difference was seen for the mutation K154A. These results are consistent with interaction of HCII with the anion-binding exosite and the S1' subsite. For HCII in the presence of either heparin or dermatan sulfate, the effect of the mutations in the anion-binding exosite was exaggerated. Inhibition of wild-type thrombin, thrombin K154A, and thrombin K52E was appropriately accelerated by glycosaminoglycan. In comparison to wild-type thrombin, acceleration of inhibition rates by glycosaminoglycan was significantly less for the anion-binding exosite mutant thrombin R70E and particularly R68E. The reduction in the inhibition rate of thrombin K52E relative to wild-type thrombin was similar either in the presence or absence of glycosaminoglycan. These results suggest that the specificity of HCII, unlike ATIII, is determined by interaction with unique surface residues of thrombin. Furthermore, interaction with the anion-binding exosite, especially residue Arg-68, is required for optimal acceleration of HCII inhibition by glycosaminoglycan. As demonstrated by kinetic studies with the chromogenic substrate S-2238 and by inhibition assays with ATIII, these mutations do not affect the active site directly. Therefore, the mutations probably disrupt a secondary binding site on thrombin for HCII.

Alternative explanations for the markedly slower inhibition rates observed with these mutations would be either disruption of the heparin binding site on thrombin or increased hydrolysis of HCII in the ternary complex with glycosaminoglycan (46). It is unlikely that the effect of these mutations was due to disruption of the heparin binding site on thrombin since heparin-dependent ATIII inhibition was completely unaffected. This question could be approached directly if mutagenesis could selectively inactivate the heparin-binding site on thrombin (49). Likewise, the slower inhibition rates were not due to increased cleavage of HCII by the mutant thrombins (Fig. 3). Compared to wild-type thrombin, the mutant thrombins did not cleave increased amounts of HCII prior to complex formation, nor did they show an increased rate of accumulation of the cleaved form. Thus, the slower inhibition rates for thrombins R68E and R70E were likely due to disruption of the initial binding of HCII to thrombin. Interestingly, for thrombin K52E, the accumulation of the cleaved form of HCII was decreased relative to complex formation. This suggests that the K52E mutation affects the ability of thrombin to cleave HCII, perhaps by slowing the rate of acyl-enzyme hydrolysis.

Our results suggest that the interaction of HCII with the anion-binding exosite provides the molecular basis of its specificity for thrombin, as well as a means for glycosaminoglycan to accelerate the inhibition reaction. The results are consistent with the proposed model of HCII inhibition in which the acidic domain of HCII is displaced by glycosaminoglycan (25, 26); however, they do not exclude interaction of the acidic domain of HCII with the anion-binding exosite of thrombin in the absence of glycosaminoglycan. The acidic domain of HCII may potentially be in equilibrium between unexposed and exposed conformations, and the addition of glycosaminoglycan may shift this equilibrium toward the exposed conformation. This would be consistent with the modest reductions in inhibition rates relative to wild-type thrombin seen for the exosite mutants without glycosaminoglycan; these differences in rate are exaggerated in the presence of glycosaminoglycan. The mechanism of thrombin inhibition by HCII may be analogous to hirudin, for which the acidic carboxyl-terminal domain binds to the exosite with subsequent active site inhibition by the amino-terminal apolar domain (50). HCII binding to the exosite could facilitate inhibition by approximating the two proteins in the proper orientation, or by induction of a conformational change in the active site making it more receptive to the P1 leucine. Allosteric regulation of thrombin activity by peptides bound to the exosite has been observed (51), and conformational changes in the active site following binding of thrombomodulin have been demonstrated by the use of fluorescent structural probes (52).

These results are in agreement with the recent work of Rogers et al. (45) using proteolyzed derivatives of human α-thrombin. Human γγ-thrombin is cleaved after Arg-62, Arg-73, and Lys-154; it is missing the peptide segment Ile-63 to Arg-73 in the anion-binding exosite. Compared to α-thrombin, γγ-thrombin is inhibited by HCII 2.9-fold slower without glycosaminoglycan and approximately 1,500-fold slower in the presence of heparin or dermatan sulfate. These results are consistent with the properties of thrombins R68E and R70E (Fig. 2); the larger magnitude of the differences probably results from more extensive structural changes in the proteolyzed γγ-thrombin. Human ε-thrombin, which is cleaved only at Ala-150, is inhibited by HCII only slightly more slowly than α-thrombin either in the absence (1.5-fold) or presence (2.3-fold) of glycosaminoglycan. These modest differences are consistent with the minimal effects on inhibition rate seen for the K154A mutation, located in the same surface loop of thrombin.

In conclusion, the results of this study contrast the molecular interactions of thrombin with the serpins HCII and ATIII. The specific thrombin inhibitor HCII has significant interactions with unique surface residues of thrombin in the active site cleft (Lys-52) and the anion-binding exosite (Arg-68 and Arg-70). The interaction with the anion-binding exosite, especially residue Arg-68, is critical for the acceleration of inhibition by glycosaminoglycan. This interaction with the exosite provides the postulated secondary binding site that explains the ability of HCII to inhibit thrombin despite having a P1 leucine residue. In contrast, ATIII does not have important interactions with these unique surface residues of thrombin, and mutations in the anion-binding exosite do not affect the ability of glycosaminoglycan to accelerate thrombin inhibition by ATIII. The ability of ATIII to inhibit a broad range of trypsin-like proteases may depend not only on its interaction with conserved structural motifs of these enzymes but also its failure to interact with surface residues unique to thrombin.

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