Heparin cofactor II and antithrombin are plasma serine proteinase inhibitors whose ability to inhibit α-thrombin is accelerated by glycosaminoglycans. Dysfunctional thrombin mutants Quick I (Arg167, Cys) and Quick II (Gly226→Val) were used to further compare heparin cofactor II and antithrombin interactions. Quick I, Quick II, and α-thrombin were eluted at the same salt concentration from heparin-Sepharose suggesting that the putative heparin-binding site (also termed anion binding exosite-II) is functional. Antithrombin yielded similar inhibition rates for Quick I and α-thrombin in the absence or presence of various amounts of heparin. Also, Quick I was inhibited similarly to α-thrombin by heparin cofactor II in the absence of glycosaminoglycan. In contrast, glycosaminoglycan-accelerated Quick I inhibition by heparin cofactor II was greatly reduced indicating that anion binding exosite-I (where the mutation occurs in Quick I) is critical for increased inhibition by heparin cofactor II. We also found that heparin cofactor II formed a SDS-resistant bimolecular complex with Quick II and α-thrombin at similar rates and the rate of complex formation was accelerated in the presence of glycosaminoglycans. A three-dimensional molecular model of the Quick II active site compared to α-thrombin suggested that the heparin cofactor II Leu-Ser-reactive site sequence (P1-P1′) is a compatible “pseudosubstrate” in contrast to the Arg-Ser sequence found in antithrombin. The importance of heparin cofactor II as a thrombin regulator will depend upon its ability to interact with glycosaminoglycans and the functional availability of thrombin exosites.

α-Thrombin is an important enzyme in both the activation and attenuation of hemostasis (Refs. 1–3, and references cited therein). Its reactivity is regulated by a number of proteins, including antithrombin, heparin cofactor II, and thrombomodulin (4–6). Thrombin interacts with many substrates and regulatory proteins both through the active site and through secondary binding domains, called “exosites” (2, 3, 7–9). Anion binding exosite-I (comprised of residues Arg35 to Glu60, Arg124, Lys126, Lys127, and Lys149β) has been shown to bind fibrinogen, hirudin, and thrombomodulin (2, 3, 7–15). Anion binding exosite-II (comprised of arginyl residues 93, 97, 101, 126, 165, 173, 175, and 263 and lysyl residues 169, 235, 236, and 240) is the putative heparin-binding region and is located on the surface of α-thrombin opposite anion binding exosite-I (Refs. 3 and 16, and references cited therein). Characterization of thrombin mutants and derivatives of α-thrombin generated by limited proteolysis and chemical modification has been instrumental in defining the structural and functional requirements of protein and glycosaminoglycan interactions.

Heparin cofactor II and antithrombin (historically called antithrombin III) are members of the serine proteinase inhibitor (serpin) superfamily of proteins (for a review, see Refs. 4, 5, and 17–19). Inhibition of thrombin by both serpins is greatly accelerated in the presence of the glycosaminoglycan heparin, but several differences between antithrombin and heparin cofactor II are apparent (4, 5, 17–23). Antithrombin inhibits most of the coagulation pathway enzymes and its activity can be accelerated by heparin and heparan sulfate (4). In contrast, heparin cofactor II inhibits only thrombin in coagulation and a variety of glycosaminoglycans and other polyanions are capable of enhancing this inhibition (5). The reactive-site P1-P1′ residues of heparin cofactor II are Leu-Ser, distinct from the Arg-Ser in antithrombin and most other thrombin substrates (4, 18). Additionally, heparin cofactor II possesses a distinct acidic amino-terminal domain (24). These unique structural differences suggest that antithrombin and heparin cofactor II utilize different mechanisms for thrombin inhibition. Examination of heparin cofactor II inhibition of human α-, ε-, and γ-thrombin demonstrated a requirement of both anion binding exosite-I and -II for efficient glycosaminoglycan acceleration of proteinase inhibition (25). Furthermore, studies with recombinant heparin cofactor II indicate a role for the unique acidic amino-terminal domain in the glycosaminoglycan-accelerated inhibition of thrombin (26–28). This is in contrast to antithrombin, which requires only anion binding exosite-II for accelerated inhibition of α-thrombin or its proteolyzed ε and γ derivatives (25).

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‡ The abbreviations used are: serpin, serine proteinase inhibitor; Chromozym-TH, Nα-tosyl-Gly-Pro-Arg-p-nitroanilide; FPR, d-Phe-Pro-Arg chloromethyl ketone; FPL, d-Phe-Pro-Leu chloromethyl ketone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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The work described here used the naturally occurring thrombin mutants, Quick I and II. Quick I has an Arg~Cys substitution which is located in the β-loop region of α-thrombin, part of anion binding exosite-I (15). This thrombin variant has greatly reduced fibrinogen hydrolysis, factor V activation, and platelet aggregation activity (15, 29, 30). However, Quick I does form a bimolecular complex with antithrombin, and it interacts normally with the active site-directed inhibitor disopropyl fluorophosphate (30). In Quick II, a Gly~Val substitution in the primary substrate binding pocket results in a dramatic loss of catalytic activity toward normal thrombin substrates, and inability to hydrolyze fibrinogen (31). We report that for Quick I (Arg→Cys), inhibition by antithrombin heparin is unaltered, whereas there is a dramatic difference in glycosaminoglycan-accelerated inhibition by heparin cofactor II when compared to the corresponding inhibition of α-thrombin. We further show that Quick II (Gly→Val) forms a bimolecular complex with heparin cofactor II, but not antithrombin, and that formation of the complex can be accelerated by glycosaminoglycans.

Molecular modeling of Quick II and α-thrombin suggests that differences in reactivity with heparin cofactor II and antithrombin are attributable to the difference in the serpin reactive site residues.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin cofactor II, antithrombin, and prothrombin were purified from outdated human plasma as previously described (32). α-Thrombin was generated from prothrombin and purified as described (33). γ-Thrombin was limited by proteolytic action of α-thrombin with trypsin (11). Thrombin Quick I and II were prepared from dysprothrombin Quick isolated from plasma as described previously (34). Heparin, dermatan sulfate, Polybrene, and heparin-Sepharose were obtained from Dienes BV (The Netherlands), Calbiochem, Aldrich, and Pharmacia LKB Biotechnology, respectively. Dermatan sulfate was nitric acid treated to remove contaminating heparin and heparan sulfate (35). Chromozym-TH (N-p-tosyl-Gly-Pro-Arg-pnitroanilide) was from Boehringer Mannheim.

**Thrombin Binding to Heparin**—A heparin-Sepharose column (1 × 5 cm) was loaded with either 20 nM α-thrombin, 20 nM Quick I, 20 nM Quick II, or 20 nM γ-thrombin and eluted with a linear salt gradient (100 mM to 1.2 M NaCl) using a Pharmacia fast protein liquid chromatography system. The elution profile for all but Quick I1 was determined by the absorbance of each fraction at 280 nm.

**SDS-PAGE of Serpin-Thrombin Complexes**—Serpin-thrombin covalent complexes were visualized by SDS-PAGE. Equal molar (1 μM) amounts of antithrombin or heparin cofactor II were added to α-thrombin, Quick I, or Quick II and incubated for 30 min at 37 °C. The resulting SDS-resistant complexes were separated by SDS-PAGE using the Laemmli buffer system (unreduced) and 10% polyacrylamide gels stained with Coomassie Blue (36).

**Thrombin and Thrombin Quick I Inhibition Assay**—Heparin cofactor II and antithrombin inhibition of thrombin and Quick I in the absence or presence of glycosaminoglycans was performed as described (25, 37). Briefly, heparin cofactor II (1 or 2 μM) or antithrombin (0.5 μM) was added to α-thrombin or Quick I (10 nM) in 20 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% (w/v) polyethylene glycol, 5 mg/ml bovine serum albumin, 100 μg/ml Polybrene, at 25 °C. At various time points, Chromozym-TH was added (150 nM final concentration) and the thrombin hydrolysis of this substrate was measured at 403 nm in a Vmax kinetic microplate reader ( Molecular Devices) and compared to controls (thrombin without heparin cofactor II or antithrombin). Glycosaminoglycan template experiments were performed using the concentrations of heparin and dermatan sulfate used with α-thrombin or Quick I, and 20 nM heparin cofactor II or 5 nM antithrombin. Samples were incubated for various intervals before addition of substrate with Polybrene. These experiments were performed a minimum of three times and a representative experiment is shown.

**Thrombin Quick II Inhibition Assays**—Quick II inhibition by heparin cofactor II was performed using a SDS-PAGE assay. Quick II (1.4 μM) was incubated with heparin cofactor II (14 μM) in the absence or presence of glycosaminoglycans (5 μg/ml dermatan sulfate or 10 μg/ml heparin) for various times at room temperature before quenching the reaction with 2% (w/v) SDS at 95 °C. The samples were then analyzed by SDS-PAGE. After Coomassie Blue staining, the gels were photographed and then scanned on an Apple Scanner using Apple-Scan software (Apple Computers). The rate of Quick II loss (due to the formation of the serpin-thrombin complex), a k2 value, was determined using Scan Analysis (Biosoft Inc., Cambridge, United Kingdom). This was repeated a minimum of three times and the average k2 value was reported. Control experiments with 1.4 μM α-thrombin and 14 μM heparin cofactor II gave a calculated k2 value of 3.8 × 10−5 M−1 s−1, similar to the 6 × 10−5 M−1 s−1 observed using the chromogenic substrate, Chromozym-TH (data not included).

**Molecular Modeling**—The three-dimensional coordinates of D-Phe-Pro-Arg chloromethyl ketone-inhibited α-thrombin (FPF-α-thrombin) were a kind gift of Dr. Wolfram Bode, Max-Planck-Institut für Biochemie, Martinsried, Germany (10). Modeling of Quick I and II was performed using SYBYL (Tripos Associates, St. Louis) and AMBER (Version 3.1, U. C. Singh, P. K. Weiner, J. W. Caldwell, and P. A. Kollman, Dept. of Pharmaceutical Chemistry, University of California, San Francisco, 1988). The energy of residues Gly and the arginyl residue of FPR (termed Arg in I) in FPR-α-thrombin was calculated using the TRIPPOS force field in SYBYL. The van der Waals energy component of this calculation is a measure of the van der Waals interaction between the atoms of 2 residues. For modeling of thrombin Quick II, Gly→Val of FPR-α-thrombin was changed to Val. Next, the Arg of FPR (Arg in I) was changed to a Leu and visually fitted to the specificity binding pocket in thrombin Quick II, fitting the Leu side chain relative to the Arg. The energy of Val→Leu (the mutated leucyl residue) in thrombin Quick II was calculated. The thrombin Quick I molecule was minimized in a water box using AMBER. First, 1000 cycles of Steepest Descent minimization were performed and then an additional 2700 cycles of Conjugate Gradient minimization.

**RESULTS**

**Thrombin Quick I and II Complex Formation with Serpins and Heparin Binding Affinity**—The serpins heparin cofactor II and antithrombin form essentially irreversibly covalent complexes with the proteinase thrombin. Dysthrombin characterization with heparin cofactor II and antithrombin requires defining their ability to form bimolecular complexes and to bind heparin. The complexes formed by incubation of thrombin, Quick I or II, and heparin cofactor II or antithrombin for 30 min at 37 °C were visualized by SDS-PAGE (Fig. 1, A and B). α-Thrombin (Fig. 1, A and B, lane 1; M, 36,600), Quick I (Fig. 1A, lane 2), and Quick II (Fig. 1B, lane 2) formed SDS-resistant complexes with heparin cofactor II of M, 102,000 (Fig. 1B, lanes 4–6). α-Thrombin-antithrombin and Quick I-antithrombin complexes of M, 93,000 were also observed (Fig. 1A, lanes 4 and 5). Under identical conditions, Quick II did not form a complex with antithrombin (Fig. 1A, lane 6). This suggests that the Gly→Val mutation prevents the normal formation of a bimolecular complex with antithrombin but not with heparin cofactor II or the antithrombin does not form a stable SDS-resistant complex.

To confirm that the dysthrombin mutations do not influence heparin binding, the relative heparin binding affinity of Quick I and Quick II, compared to γ-thrombin and α-thrombin, was determined. α-Thrombin, Quick I, Quick II, and γ-thrombin were loaded separately onto a heparin-Sepharose affinity column and eluted with a linear salt gradient (Fig. 2). All of the thrombins eluted at a similar salt concentration (0.6 M; Fig. 2). This suggests the heparin-binding regions of α-thrombin, Quick I, Quick II, and γ-thrombin are functionally indistinguishable under these conditions regardless of the protein modifications.

**Antithrombin and Heparin Cofactor II Inhibition of Thrombin Quick I**—The formation of a SDS-resistant complex in Fig. 1 indicates that Quick I interacts with antithrombin and
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Heparin-Sepharose affinity chromatography. α-Thrombin (O), γ,γ-thrombin (C), or Quick I (■) were loaded onto a heparin-Sepharose fast protein liquid chromatography affinity column and eluted with a linear salt gradient from 10 mM to 1.2 M NaCl. Eluted fractions were assayed for chromogenic activity and the absorbance at 280 nm determined. Quick II was loaded onto the same column and its peak of elution (¶) was determined by measuring the absorbance at 280 nm.

The rate of inhibition of Quick I by heparin cofactor II was only slightly reduced relative to α-thrombin in the absence of glycosaminoglycans (Table I). In contrast, in the presence of heparin or dermatan sulfate, heparin cofactor II inhibited Quick I 220- and 625-fold slower than α-thrombin, respectively (Table I). Maximal inhibition of α-thrombin and Quick I occurs at 200 μg/ml for heparin (Fig. 4, top panel), 1.0 and 4.0 mg/ml, respectively, for dermatan sulfate (Fig. 4, bottom panel). This observation suggests that the Arg residue at position 67 in the β-loop region of anion binding exosite-I is important in the glycosaminoglycan-accelerated inhibition of thrombin by heparin cofactor II. Arg67 is not required for heparin-accelerated inhibition of thrombin by antithrombin.

**FIG. 3.** Heparin-dependent inhibition of thrombin by antithrombin. Hydrolysis of Chromozym-TH was determined for α-thrombin (■) or Quick I (●) in the presence of 10-fold molar excess antithrombin and increasing concentrations of heparin. The apparent inhibition rate constant was calculated as $K_{i}$ at 25°C. A representative experiment is shown.

**TABLE I**

<table>
<thead>
<tr>
<th>Serpin/glycosaminoglycan</th>
<th>Inhibition rate constant ($k_2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>0.6</td>
</tr>
<tr>
<td>HC</td>
<td>0.06</td>
</tr>
<tr>
<td>AT/heparin</td>
<td>1900</td>
</tr>
<tr>
<td>HC/heparin</td>
<td>750</td>
</tr>
<tr>
<td>AT/dermatan sulfate</td>
<td>1200</td>
</tr>
<tr>
<td>AT/antithrombin</td>
<td>390</td>
</tr>
<tr>
<td>AT/Quick I</td>
<td>750</td>
</tr>
<tr>
<td>AT/Quick II</td>
<td>1200</td>
</tr>
<tr>
<td>AT/Quick II/AT</td>
<td>1.2</td>
</tr>
<tr>
<td>AT/Quick II/HC</td>
<td>2.3</td>
</tr>
<tr>
<td>AT/Quick II/HC/AT</td>
<td>2.3</td>
</tr>
<tr>
<td>AT/Quick II/HC/AT</td>
<td>2.3</td>
</tr>
<tr>
<td>AT/Quick II/HC/AT/HC</td>
<td>2.3</td>
</tr>
<tr>
<td>AT/Quick II/HC/AT/HC/AT</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The inhibition rate constants presented were determined from the mean of three separate experiments.

Indicates the -fold difference between the calculated rate for α-thrombin and Quick I.

Heparin concentrations of 1 and 5 μg/ml, respectively.

Dermatan sulfate concentrations of 1 and 4 mg/ml, respectively.

It has been suggested that Quick II does not react with normal α-thrombin arginyl-containing substrates because the Gly238 to Val mutation either blocks access to the specificity binding pocket or causes other structural changes within the pocket (3, 31, 38). Our results suggest that Quick II does react with heparin cofactor II, a leucyl-containing substrate. To investigate this hypothesis further we used computer-assisted
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three-dimensional molecular modeling. In addition to the stereo models, the interaction between α-thrombin or Quick II with Arg- or Leu-containing substrates was further evaluated by calculating the van der Waals interaction energy and closest distances between these residues. The van der Waals energy component of the total energy calculation depends only on the interactions between the atoms of the 2 residues. Therefore, the interaction energies can be used as a qualitative measure of the steric fit of these residues but not for an exact comparison of the different models. The interaction of α-thrombin with an arginyl-containing substrate, such as antithrombin, was modeled using the active-site region of FPR-α-thrombin. The van der Waals radii of the Gly to Val mutation in α-thrombin are consistent with our inhibition assays using heparin cofactor II and antithrombin.

**DISCUSSION**

This study was undertaken in order to better understand how thrombin interacts with heparin cofactor II and antithrombin. Our results focus on two dysfunctional thrombins, Quick I and Quick II, and how the specific regions of the mutant proteins are involved in the physiological regulation of thrombin by heparin cofactor II and antithrombin. The serpin heparin cofactor II specifically inhibits thrombin, and inhibition is accelerated by the glycosaminoglycans heparin and dermatan sulfate. The mechanism of heparin cofactor II/glycosaminoglycan inhibition of thrombin differs from that described for the major blood plasma thrombin inhibitor, antithrombin (25-28). Heparin cofactor II contains an acidic amino-terminal domain that is not found in other serpin family members. This region of the protein is necessary for optimal glycosaminoglycan-mediated acceleration of thrombin inhibition. Mutagenesis studies of heparin cofactor II by both van Deerlin and Tollefsen (26) and Ragg et al. (27, 28) suggest that changing residues in this acidic amino terminator to basic residues decreases the acceleration of inhibition in the presence of glycosaminoglycans. The acidic amino terminus of heparin cofactor II is proposed to interact with anion binding exosite-I in α-thrombin. This hypothesis is further supported by the work of Rogers et al. (25), where inhibition of thrombin by heparin cofactor II in the presence of glycosaminoglycans is reduced with γI-thrombin, a proteolyzed thrombin molecule missing most of anion binding exosite-I, specifically, the β-loop region. The current model of heparin cofactor II interaction with thrombin suggests that the glycosaminoglycan bridges the glycosaminoglycan-binding regions of heparin cofactor II and thrombin, the acidic amino terminus of heparin cofactor II is displaced and is then able to interact with anion binding exosite-I of thrombin (25-28). Thus, collective stabilization ("double bridging") of the interaction between heparin cofactor II and thrombin by the glycosaminoglycan and the amino terminus of heparin cofactor II results in the dramatic acceleration of inhibition.

Thrombin Quick I possesses a single amino acid substitution (Arg→Cys) in the β-loop region of anion binding exosite-I (15). It reacts normally with antithrombin and heparin cofactor II and binds heparin with the same affinity as α-thrombin. Heparin cofactor II inhibition of Quick I relative to α-thrombin is essentially normal in the absence of glycosaminoglycans. In the presence of heparin or dermatan sulfate, however, the rate enhancement for Quick I inhibition is reduced relative to that for α-thrombin. This is in contrast to antithrombin where the rate of inhibition of α-thrombin and Quick I is the same in the presence or absence of heparin. Therefore, a different mechanism must be involved in the
Molecular modeling of α-thrombin and Quick II with a P1 Leu substrate. Computer-assisted three-dimensional molecular modeling (stereo views) of the substrate binding pocket of α-thrombin with FPR (top panel) and Quick II with FPL (bottom panel). α-Thrombin and Quick II residues are labeled on the right with the substrate P1 Arg residue (Arm41) and Leu (Lem41) labels on the left.

FIG. 5. Molecular modeling of α-thrombin and Quick II with a P1 Leu substrate. Computer-assisted three-dimensional molecular modeling (stereo views) of the substrate binding pocket of α-thrombin with FPR (top panel) and Quick II with FPL (bottom panel). α-Thrombin and Quick II residues are labeled on the right with the substrate P1 Arg residue (Arm41) and Leu (Lem41) labels on the left.

glycosaminoglycan-accelerated inhibition of thrombin by heparin cofactor II compared to antithrombin. Our results are consistent with the β-loop region of Quick I participating in heparin cofactor II inhibition of thrombin in the presence of glycosaminoglycans.

The Arg⁶⁷ to Cys substitution in Quick I must result in a structural change in the β-loop region of the thrombin molecule. Normally, Arg⁶⁷ and Glu⁶⁸ form a salt bridge, which presumably stabilizes this region of the molecule (10). The Cys at residue 67 in the variant thrombin cannot form this interaction. The resulting structure of this domain can be predicted by computer-assisted molecular modeling where the thrombin molecule was minimized upon substituting Cys for Arg⁶⁷ (Fig. 6). The top panel is a stereo view of the α-thrombin β-loop region and the bottom panel is the same region of Quick I. The Cys permits more flexibility to the region and probably alters the β-loop conformation such that efficient interaction with heparin cofactor II in the presence of glycosaminoglycan does not occur.

The results presented here also demonstrate heparin cofactor II’s ability to interact with Quick II where other “classic” thrombin substrates fail. Quick II has dramatically impaired catalytic activity toward normal thrombin substrates (30, 31). It has 10³-fold lower affinity for hirudin, although it binds hirudin lacking the amino-terminal residues only 16-fold less than α-thrombin (38). Quick II also binds platelets with reduced affinity (39). Henriksen and Mann (31) previously demonstrated that Quick II was able to inefficiently hydrolyze
the elastase substrate succinyl-Ala-Ala-Pro-Leu-p-nitroanilide. Our results demonstrate that Quick II appears to bind heparin normally, but it does not interact with the thrombin chromogenic substrate or form a complex with antithrombin. We observe that heparin cofactor II-Quick II complex formation can be accelerated by glycosaminoglycans. The observation that heparin cofactor II forms a complex with Quick II which is stable in SDS can be better understood by molecular modeling which suggests that the reactive-site P1 Leu (Leu-Ser) of heparin cofactor II would not extend as far into the primary substrate binding pocket as a normal thrombin substrate possessing an Arg in the P1 position (Arg-Ser). The Val substitution in Quick II is postulated to disturb the conformation of the specificity pocket in part by disrupting Asp\(^{38}\) (3, 38). The Asp\(^{38}\) carboxylate group would normally interact ionically with the P1 Arg guanidinium group to contribute to the specificity of the Arg-Ser substrate. It has also been concluded that the Quick II mutation disrupts surface residues resulting in its reduced affinity for platelets and hirudin (38, 39).

In conclusion, heparin cofactor II clearly differs from antithrombin during thrombin regulation and inhibition, especially in the presence of glycosaminoglycans. The mechanism of glycosaminoglycan acceleration for thrombin inhibition by heparin cofactor II is consistent with ternary complex formation which then makes available the unique acidic domain in heparin cofactor II for interaction with the \(\beta\)-loop region of thrombin. Furthermore, subtle distinctions must be present in the heparin cofactor II reactive-site domain compared to that in antithrombin for thrombin active-site recognition. A question that remains is how these thrombin exosites participate in vivo during inhibition. It is likely that cellular and extracellular matrix thrombin interactions involved in localization of the proteinase are important for directing physiological inhibition of thrombin by heparin cofactor II and antithrombin and the acceleration of inhibition by glycosaminoglycans.

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