Role of Thrombin Exosites in Inhibition by Heparin Cofactor II

Susannah J. Rogers, Charlotte W. Pratt, Herbert C. Whinna, and Frank C. Church

From the Center for Thrombosis and Hemostasis and Departments of Pathology and Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7035

We determined the role of specific thrombin “exosites” in the mechanism of inhibition by the plasma serine proteinase inhibitors heparin cofactor II (HC) and antithrombin (AT) in the absence and presence of a glycosaminoglycan by comparing the inhibition of α-thrombin to ε- and γ1-thrombin (produced by partial proteolysis of α-thrombin by elastase and trypsin, respectively). The thrombin derivatives were inhibited in a similar manner by AT, either in the absence or presence of heparin, which confirmed the integrity of both heparin binding abilities and serpin reactivities of ε- and γ1-thrombin compared to α-thrombin. Antithrombin activities of HC in the absence of a glycosaminoglycan with α-, ε-, and γ1-thrombin were similar with rate constants of 3.5, 2.4, and 1.2 × 10^7 M^-1 min^-1, respectively. Interestingly, in the presence of glycosaminoglycans the maximal inhibition rate constants by HC with heparin and dermatan sulfate, respectively, were as follows: 30.0 × 10^7 and 60.5 × 10^7 for α-thrombin, 14.6 × 10^7 and 24.3 × 10^7 for ε-thrombin, and 0.017 × 10^7 and 0.034 × 10^7 M^-1 min^-1 for γ1-thrombin. A hirudin carboxyl-terminal peptide, which binds to anion-binding exosite-I of α-thrombin, dramatically reduced α-thrombin inhibition by HC in the presence of heparin but not in its absence. We analyzed our results in relation to the recently determined x-ray structure of D-Phe-Pro-Arg-chloromethyl ketone-α-thrombin (Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475). Our results suggest that the β-loop region of anion-binding exosite-I in α-thrombin, which is not present in γ1-thrombin, is essential for the rapid inhibition reaction by HC in the presence of a glycosaminoglycan. Therefore, α-thrombin and its derivatives would be recognized and inhibited differently by HC and AT in the presence of a glycosaminoglycan.

α-Thrombin is a trypsin-like serine proteinase important for hemostasis (1, 2 and references cited therein). It hydrolyzes fibrinogen to fibrin in the final step of blood coagulation and interacts with other substrates, receptors, and inhibitors (1, 2). The three-dimensional structure of thrombin reveals that it is similar to related pancreatic serine proteinases but that it has a series of exposed insertion loops (3–5). Physiological substrate recognition by α-thrombin is mediated by both the active site and separate secondary binding sites, termed “exosites” (1–5). Different forms of thrombin can be produced by limited proteolysis using elastase or trypsin to yield ε- and γ1-thrombin, respectively (6–9). ε-Thrombin is cleaved at Ala-149A and has partially reduced fibrinogen clotting activity (6–8). γ1-Thrombin has lost virtually all fibrinogen clotting activity, and the cleavage sites are Lys-149E, Arg-67, and Arg-77A (1, 2, 8, 9). The region of thrombin containing Ala-149A and Lys-149E is termed the autolysis loop and is comprised of Lys-146 to Gly-150 (1–3, 8). The region between Arg-67 to Arg-77A contains a large number of basic residues and is termed the β-loop (1–3, 8). Thus, γ1-thrombin is altered only in the autolysis loop while γ1-thrombin is cleaved in the autolysis loop and the β-loop is absent.

One mechanism for the in vivo regulation of thrombin is inhibition by the serine proteinase inhibitors (serpin)2 antithrombin (AT), and heparin cofactor II (HC) (10–14). HC and AT form essentially irreversible 1:1 complexes with thrombin. The rate of thrombin inhibition by AT and HC is dramatically increased by the presence of glycosaminoglycans such as heparin (AT and HC) and dermatan sulfate (HC) (10–19). The glycosaminoglycan-enhanced thrombin inhibition by AT and HC requires simultaneous binding of proteinase and serpin to glycosaminoglycan, which suggests that a “bridging” effect is necessary for accelerated inhibition (15–19).

The specificity of HC appears to be largely limited to thrombin and chymotrypsin (20–22) while AT inhibits most of the proteinases in the intrinsic coagulation pathway (14). An intriguing difference in the two serpins is the reactive site: Leu-Ser in HC (12, 23) and the expected Arg-Ser in AT (14). The occurrence of the reactive site Leu in HC raises speculation that residues far separated in primary structure may be crucial for recognition between thrombin and HC. Recent experimental evidence using HC mutants supports a hypothesis that an additional acidic region of HC (far removed in primary structure from the reactive site) is partially responsible for the accelerated thrombin inhibition reaction in the presence of a glycosaminoglycan (24–26).

In this study we explored whether or not different thrombin...
exosites, which are altered or missing in three of the four derivatives (human α-, ε-, and γT-thrombin and bovine α-thrombin (with occurrence of Glu for Lys-149E)), play a role in the mechanism of glycosaminoglycan-enhanced inhibition by HC or AT. Specifically, we were interested in the exosite termed "anion-binding exosite-I" (an electropositive surface formed by Arg-35 to Glu-39, Arg-67 to Arg-77A, and Lys-149E) which is critical for α-thrombin interactions with fibrinogen, hirudin, and thrombomodulin (1-5, 8, 27, 28). Whereas there was no requirement for these α-thrombin exosites during the AT/heparin inhibition reaction, we found that the β-loop region of anion-binding exosite-I was essential for rapid inhibition by HC in the presence of glycosaminoglycans.

EXPERIMENTAL PROCEDURES

Materials—Human plasmin HC and AT were purified as described previously (11). Heparin was obtained courtesy of Diosynth BV, Oss, the Netherlands; dermatan sulfate from Calbiochem was treated with nitric acid to remove heparin and heparan sulfate contaminants (29). Polybrene was from Aldrich; the thrombin substrate Chromozym TH (N'-p-tosyl-Gly-Pro-Arg-p-nitroanilide) was obtained from Boehringer Mannheim. Trypsin (tosylphenylalanine chloromethyl ketone treated) and porcine pancreatic elastase were purchased from Sigma and Calbiochem, respectively. The synthetic hirudin peptide from residue 53 to 64 (hirudin53-64; NGDFEEIPEEYL with an unsulfated Tyr-63) and a control peptide (YGHRPLDKKREEAPSLR) from residue 53 to 66 were synthesized as detailed elsewhere (31). Except where noted, all inhibition experiments were performed in 20 mM HEPES, 150 mM NaCl, pH 7.4, at 25 °C.

Thrombin Derivatives—Human (Hu) and bovine (Bv) α-thrombin were purified as described previously (32). Human γT-thrombin and ε-thrombin were prepared by limited proteolysis of α-thrombin with trypsin (8) and elastase (6), respectively. The proteins were active site-titrated with p'-guanidinobenzoate (33). All of the thrombin derivatives were greater than 95% active by active site titration. Fibrinogen clotting activity of the thrombin derivatives was performed as detailed previously using a V_m kinetic microparticle reader in the kinetic mode (Molecular Devices, Menlo Park, CA) (30). The ε- and γT-thrombin derivatives had 53% and 1% of the fibrinogen clotting activity, respectively, compared to Hua-thrombin. NADdSO4-polyacrylamide gel electrophoresis was performed in 15% slab gels (34) without chemical reduction. NaDodS04-polyacrylamide gels were stained by Coomassie Blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the thrombin derivatives revealed three major components in γT-thrombin and only two components in ε-thrombin as reported previously (6, 8).

Thrombin Inhibition Assays—Antithrombin inhibition assays in the absence or presence of glycosaminoglycans with HC, AT, and the thrombin derivatives were performed as described (22, 25). In the absence of a glycosaminoglycan, the thrombin derivatives (10 nM) were preincubated with HC (1 μM) or AT (500 nM) in a final volume of 100 μl with 100 μg/ml polybrene present. In the presence of a glycosaminoglycan, the thrombin derivatives (0.5 nM) and AT (5 nM) were incubated in a final volume of 100 μl with various concentrations of heparin or dermatan sulfate. For both assay procedures, Chromozym TH with polybrene was added at various time intervals. Chromozym TH hydrolysis was measured in a V_m kinetic microparticle reader and compared to thrombin controls (same reaction components minus serpin). All thrombin inhibition experiments were performed at least three times and the results averaged.

Huo-thrombin inhibition by HC alone in the presence of hirudin53-64 was performed in the HEPES-buffered saline with 0.1 mg/ml bovine serum albumin as detailed (30). Briefly, Huo-thrombin (5 nM) was preincubated with HC (500 nM), and without 750 nM hirudin53-64, and remaining thrombin activity was measured with Chromozym TH. This experiment was performed three times and the results averaged. Huo-thrombin inhibition by HC/heparin in the presence of hirudin53-64 or the control peptide was performed by incubating the active site-specific inhibitor DAPA using the reaction conditions as detailed previously (36). Huo-thrombin and DAPA were mixed to final concentrations of 50 and 450 nM, respectively, in the absence and presence of 6.25 μM hirudin53-64 or the control peptide, and a solution of HC/heparin (stock of 8 μM HC and 10 μg/ml heparin) was added to the DAPA-thrombin solution. The reaction was monitored by following the loss of the DAPA-thrombin fluorescence as described previously (36). This experiment was performed eight times and the results averaged.

Molecular Modeling—The three-dimensional coordinates of 14-Phe-Pro-Arg-chloromethyl ketone (PPACK)-α-thrombin (3) were a kind gift of Dr. Wolfram Bode, Max-Planck-Institut für Biochemie, Martinsried, Germany. The PPACK-α-thrombin structure was modeled using the SYBYL software package from Tripos Associates and drawings were made using the Tripos program NITRO on a Macintosh II.

RESULTS

Inhibition by Antithrombin—We measured the inhibition of the thrombin derivatives by AT both in the absence and presence of heparin. In the absence of heparin, we found that ε- and Bvo-thrombin were inhibited by AT at about the same rate (~1.5-fold less) as Huα-thrombin, while the inhibition of γT-thrombin was reduced by 6.3-fold compared to Huα-thrombin (Table I). All of the thrombin derivatives showed a typical bell-shaped inhibition rate curve with AT as a function of heparin concentration, with maximal inhibition rate constants of 11.5, 10.9, 4.66, and 13.8 × 10^3 M^-1 min^-1 for Huα-, ε-, γT-, and Bvo-thrombin, respectively (data not shown). Therefore, AT inhibition of Huα-thrombin was 1.4-fold slower than that for Bvo-thrombin at the optimal heparin concentration, while the rates of ε- and γT-thrombin inhibition were reduced by 1.1- and 2.5-fold, respectively, compared to Huα-thrombin.

These data agree well with previously reported values (9, 28, 36) and the results indicate that the thrombin derivatives are inhibited similarly by AT either in the absence or the presence of heparin. Thus, it seems that neither of the regions proteolyzed in ε- or γT-thrombin nor the substitution of Glu for Lys-149E in Bvo-thrombin are of major importance for AT recognition or for heparin binding in α-thrombin.

Inhibition by Heparin Cofactor II—We examined the inhibition of the thrombin derivatives by HC in the absence and presence of the glycosaminoglycans heparin and dermatan sulfate. In the absence of a glycosaminoglycan, we found that inhibition of ε- and γT-thrombin by HC was slightly reduced by 1.5- and 2.9-fold, respectively, compared to Huα-thrombin (Table I). HC inhibition of Bvo-thrombin was 3.8-fold slower than that of Huα-thrombin (Table I). These results indicate that recognition of the thrombin derivatives by HC in the absence of a glycosaminoglycan does not depend on exosite domain(s) missing or altered in these thrombins.

As shown in Fig. 1, all of the thrombin derivatives exhibited typical bell-shape inhibition rate curves by HC as a function of glycosaminoglycan concentration. The maximal HC inhibition rate constants with heparin and dermatan sulfate were 30.0 × 10^3 and 60.5 × 10^3 for Huα-thrombin, 14.6 × 10^3 and 24.3 × 10^3 for ε-thrombin, and 0.017 × 10^3 and 0.034 × 10^3 M^-1 min^-1 for γT-thrombin, respectively (Fig. 1). The maximal inhibition rate constant of Bvo-thrombin by HC in the presence of heparin was 8.40 × 10^3 M^-1 min^-1 (data not included). Compared to Huα-thrombin, HC inhibition rates of ε- and

| Table I |
|---------|
| Inhibition of thrombin derivatives by heparin cofactor II and antithrombin in the absence of a glycosaminoglycan |
| Serpin | 
| Human α | Human ε | Human γT | Bovine α |
|---------|
| Thrombin derivative (k_D × 10^3 M^-1 min^-1) | |
| AT | 45 ± 3 | 28 ± 2 | 7.2 ± 4 | 31 ± 1 |
| HC | 3.5 ± 1 | 2.4 ± 1 | 1.2 ± 2 | 0.93 ± 0.04 |

* Rate constants for inhibition of thrombin derivatives by AT and HC were determined as described under "Experimental Procedures."
Bγa-thrombin in the presence of optimal concentrations of glycosaminoglycans were reduced by 2.3- and 3.6-fold, respectively. γγ-Thrombin inhibition by HC in the presence of heparin and dermatan sulfate was reduced by 1765- and 1800-fold, respectively, compared to Hua-thrombin. The differences found in the maximal inhibition rates between Hua-, ε-, and Bγa-thrombin derivatives in the presence of glycosaminoglycans are consistent with the values determined for inhibition by HC in the absence of a glycosaminoglycan. In contrast, the values found for γγ-thrombin inhibition by HC in the presence of a glycosaminoglycan are greatly reduced compared to Hua-thrombin suggesting that an exosite absent or altered in γγ-thrombin is necessary for rapid proteinase inhibition. Since ε- and Bγa-thrombin (both altered in the autolysis loop) have essentially normal glycosaminoglycan-enhanced inhibition by HC, these results suggest that the β-loop of anion-binding exosite-I of α-thrombin is involved in an additional interaction with HC when glycosaminoglycan is present.

To further investigate the role of anion-binding exosite-I in α-thrombin, we determined the rate of inhibition by HC (with and without heparin) in the presence of a hirudin carboxyl-terminal peptide (residues 53–64). Hirudin53-64 binds to anion-binding exosite-I to block fibrinogen recognition without a significant detrimental effect on the active site of α-thrombin (4, 5, 30, 37–40). Furthermore, we and others have reported that hirudin53-64 does not adversely influence AT inhibition of α-thrombin either in the absence or presence of heparin (30, 40). In the absence of heparin, the rate of Hua-thrombin inhibition by HC in the absence and presence of hirudin53-64 (150-fold molar excess of peptide to thrombin) was the same, 3.8 ± 0.1 × 10^4 M^{-1} min^{-1}. However, we found that hirudin53-64 reduced the rate of Hua-thrombin inhibition by HC/heparin, as shown by the inhibition rates of 1.32 ± 0.13 × 10^4 in the absence of peptide and 0.78 ± 0.11 × 10^4 M^{-1} min^{-1} in the presence of a 125-fold molar excess of peptide to thrombin (Fig. 2). The control peptide had no effect on Hua-thrombin inhibition by HC/heparin under the same conditions. Thus, in the presence of hirudin53-64 at this concentration, Hua-thrombin inhibition by HC/heparin is reduced by more than 40%. This result implies further that anion-binding exosite-I of α-thrombin is necessary for rapid inhibition by HC in the presence of a glycosaminoglycan.

**Discussion**

This study was undertaken to determine whether specific exosites of α-thrombin participate in recognition of HC and AT in either the absence or presence of a glycosaminoglycan. α-Thrombin derivatives have been previously used to probe the role of secondary binding sites during the interaction with the physiological substrate fibrinogen, the leech antithrombin protein hirudin, and the cell-surface receptor thrombomodulin (1, 2, 8, 28, 41). Heparin-binding properties of various α-thrombin derivatives have also been investigated (9, 36, 42). The usefulness of these thrombin derivatives is enhanced due to the recently determined crystal structures of PPACK-thrombin and thrombin-hirudin complex (3–5). A three-dimensional view of α-thrombin is shown in Fig. 3. There is now evidence to describe two anion-binding exosites in α-thrombin (2). Anion-binding exosite-I (see Introduction) is important for interaction with fibrinogen, the carboxyl terminus of hirudin, and thrombomodulin (1, 2). "Anion-binding exosite-II" is an electropositive surface comprised of Lys-175, Arg-233, Lys-236, and Lys-240 and is the putative heparin-binding site (5, 36).

Our results demonstrate that thrombin recognition of AT and HC in the absence of a glycosaminoglycan is similar for all of the thrombin derivatives. The small differences in inhibition of the thrombin derivatives by AT and HC indicate that neither the autolysis loop nor the β-loop are essential for proteinase interaction with serpin alone. The AT/heparin reaction is also not dramatically altered when the various thrombins are compared. This further suggests the importance of anion-binding exosite-II, present in all of these thrombin derivatives, during formation of the heparin bridge between AT and thrombin (Fig. 3).

An exciting finding is the difference in inhibition of the thrombin derivatives by HC in the presence of a glycosaminoglycan. Similar to AT/heparin, inhibition rates of Hua-, ε-, and Bγa-thrombin by HC/glycosaminoglycan are quite comparable. This indicates that the autolysis loop and Lys-149E of anion-binding exosite-I are not critical for rapid thrombin inhibition by HC in the presence of a glycosaminoglycan. However, γγ-thrombin inhibition by HC/glycosaminoglycan is greatly reduced. This large difference in inhibition
shown that a synthetic HC peptide (residues 54–75) inhibits fibrinogen clotting activity of thrombin, and the peptide competes with hirudin for binding to thrombin. These results imply that the HC acidic domain interacts with an exposed basic region of α-thrombin.

Our collective results (24–26, 43) suggest a plausible mechanism for α-thrombin inhibition by HC in the presence of a glycosaminoglycan: (i) heparin/dermatan sulfate binds to the glycosaminoglycan-binding site of HC and anion-binding exosite-II of thrombin forming a bridge (or ternary complex) similar to that for AT/heparin/thrombin; and (ii) the displaced HC acidic domain interacts with the β-loop region of anion-binding exosite-I in thrombin, which facilitates rapid proteinase inhibition by HC. Therefore, thrombin inhibition by HC in the presence of a glycosaminoglycan is consistent with a “double-bridge” mechanism in which anion-binding exosite-I of α-thrombin binds to the acidic region of HC and anion-binding exosite-II (through the glycosaminoglycan) binds to the glycosaminoglycan-binding domain of HC.

α-Thrombin has an essential role in the processes of hemostasis and wound healing which is displayed through enzymatic and nonenzymatic action by the proteinase (1, 2). Proteolyzed forms of α-thrombin have been proposed to exist in vivo and are involved in either physiological or pathological events (1, 2). Generation of α-thrombin derivatives with altered exosomes (and changed biological properties) is possible through the proteolytic action of mast cell tryptase, neutrophil elastase, and cathepsin G (1, 2). As has been shown here, these thrombin derivatives would react differently with HC and AT in the presence of a glycosaminoglycan. In vivo targeting of thrombin inhibition either by HC/glycosaminoglycan or by AT/glycosaminoglycan could be regulated by the competency of α-thrombin exosomes, particularly anion-binding exosite-I.

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We found previously that modification of lysyl residues in the glycosaminoglycan-binding site of HC (with the negatively charged penta-peptide pyridoxal 5’-phosphate) results in increased Hua-thrombin inhibition activity in the absence of a glycosaminoglycan (35). Thus, we measured inhibition of the HUA-thrombin derivatives with control and pyridoxal 5’-phosphate-modified HC. The inhibition rate constant (kobs = × 10−7 min−1) for control and modified HC were 45 ± 2 and 87 ± 1 for Hua-thrombin, 34 ± 4 and 68 ± 1 for α-thrombin, and 19 ± 2 and 20 ± 5 for γ-thrombin, respectively. The modified HC had increased antithrombin activity by 1.9-fold with Hua- and α-thrombin compared to control HC but there was no change in the inhibition rate constant between control and modified HC with γ-thrombin. This experiment implies that the modified glycosaminoglycan-binding site of HC has partially displaced the HC acidic domain, probably by charge repulsion, which slightly enhances thrombin inhibition in the absence of a glycosaminoglycan.
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